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DOCTORAL DISSERTATION

Cadmium-induced effects on the ethylene pathway and the link with oxidative stress in *Arabidopsis thaliana*

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Voorwoord

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Summary

Elevated concentrations of toxic metals such as cadmium (Cd) in soils, primarily caused by anthropogenic sources (e.g. mining), are a severe problem worldwide. Plants grown on contaminated soils accumulate Cd, which consequently enters the food chain, eliciting a major threat to the public health. In plants, Cd disturbs several developmental (e.g. growth) and physiological (e.g. photosynthesis) processes. Despite its non redox-active character, Cd is also capable of inducing the production of reactive oxygen species (ROS) at the cellular level, resulting in an oxidative challenge. Excessive ROS react with virtually all biomolecules, causing cellular damage. However, controlled levels of ROS, maintained by the antioxidative defence system, act as signal transduction molecules contributing to plant acclimation to abiotic stress such as exposure to Cd. Increasing evidence suggests an existing relation between cellular redox signalling and phytohormones, key regulators of plant growth and development, in order to control defence responses. Furthermore, the phytohormone ethylene, often considered as the 'stress hormone', is known to mediate hormone and redox signalling processes during abiotic stress. The stress-induced oxidative burst as well as the biosynthesis of glutathione (GSH), an important antioxidant during Cd stress, were already shown to be mediated by ethylene. Therefore, the aim of the current work was to unravel the involvement of ethylene biosynthesis and signalling during the Cd-induced oxidative challenge induced by sublethal Cd concentrations (5 and 10 µM Cd) in *Arabidopsis thaliana*.

Many studies have investigated the effects of Cd exposure on the biosynthesis of ethylene in various plant species, without focussing on the underlying molecular mechanisms. Accordingly, we unravelled the molecular mechanisms of enhanced ethylene biosynthesis after short-term (24 and 72 h) Cd exposure **(Chapter 3)**. Increased ethylene release was measured in wild-type (WT) *A. thaliana* plants after exposure to Cd. Enhanced mRNA levels of different ethylene responsive genes, although transiently, as well as increased concentrations of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) supported these findings. ACC synthesis by the enzyme ACC synthase (ACS) covers the rate-limiting step in ethylene biosynthesis. The transcript levels of 2 ACS isoforms, *ACS2* and *ACS6* were the most abundant ones after Cd exposure, suggesting

their importance in the Cd-induced ethylene production. This was confirmed by lower ethylene levels in *acs2-1acs6-1* double knockout (KO) mutants, resulting in a diminished fast induction of the expressions of ethylene responsive genes.

The relation between the transient Cd-induced ethylene response and the oxidative challenge was investigated by comparing responses in roots and leaves of WT and *acs2-1acs6-1* double KO-mutants **(Chapter 4)**. Lower transcript levels of pro-oxidative and oxidative stress hallmark genes together with decreased GSH levels in the leaves during short-term moderate (5 µM) Cd exposure in the mutant plants indicate a reduced oxidative challenge compared to the WT. Moreover, the mutant plants had a higher leaf fresh weight after 72 h Cd exposure. However, severe (10 µM) Cd stress seemed to overwhelm the plants, overruling most of the different responses between WT and mutant plants. We hypothesise that severe stress conditions activate multiple signalling systems, e.g. oxylipins and jasmonates, creating bypass mechanisms for ethylene signalling. Furthermore, long-term exposure inhibited the development and reproduction capacity of WT and mutant plants in a similar way, indicating that ethylene biosynthesis plays an important role in the early oxidative challenge induced by moderate Cd stress.

Based on our previous results, the early involvement of ethylene in the Cd-induced oxidative challenge was investigated by increasing our experimental resolution **(Chapter 5)**. Responses in the leaves of WT *A. thaliana* plants after short-term exposure to moderate (5 µM) Cd concentrations were compared to those in mutant plants with an impaired ethylene signal transduction pathway: *ethylene resistant* (*etr*)*1-1* (receptor), *ethylene insensitive* (*ein*)*2-1* (signal transducer) and *ein3-1* (transcription factor). A reduced oxidative challenge compared to the WT, resulting in a decreased growth inhibition by Cd stress, was observed in *etr1-1* and *ein2-1*, but not in *ein3-1* mutants. Both *etr1-1* and *ein2-1* plants showed a delayed response in the GSH metabolism, including GSH levels and transcript levels of GSH synthesising and recycling enzymes. Moreover, lower expressions of different oxidative stress hallmark genes were measured in the *ein2-1* mutants compared to the WT, evincing that ethylene signalling is also involved in the early responses to Cd stress.

In conclusion, our results support the involvement of ethylene biosynthesis and signalling in fine-tuning the early Cd-induced oxidative challenge in *A. thaliana* leaves exposed to moderate Cd concentrations.

Samenvatting

Verhoogde gehaltes aan toxische metalen zoals cadmium (Cd) in bodems zijn voornamelijk veroorzaakt door menselijk activiteiten zoals mijnbouw en vormen een wereldwijd probleem. Planten die op deze verontreinigde bodems groeien, stapelen Cd op en via consumptie van dit gecontamineerd plantaardig voedsel komt Cd vervolgens in de voedselketen terecht. Hierdoor vormt het een ernstige bedreiging voor de volksgezondheid. Planten blootgesteld aan Cd ondervinden verstoringen in verschillende fysiologische processen (vb. fotosynthese) met een verminderde groei tot gevolg. Hoewel Cd geen redox-actieve eigenschappen bezit, veroorzaakt het toch een toename van reactieve zuurstofvormen (ROS) op cellulair niveau. Een overmaat aan ROS kan enerzijds oxidatieve schade toebrengen aan verschillende cellulaire componenten, maar anderzijds zijn het belangrijke signaalmoleculen die bijdragen tot de bescherming van de plant tegen abiotische stress zoals Cd. Activatie van het antioxidatief verdedigingssysteem zorgt voor het behoud van de hoeveelheid ROS binnen fysiologische grenzen. Uit recent onderzoek blijkt dat er een relatie bestaat tussen redox-signalisatie en planthormonen in de verdediging van de plant tegen stress. Daarnaast zijn deze planthormonen ook belangrijk tijdens de groei en ontwikkeling van de plant. Ethyleen wordt vaak beschouwd als stresshormoon en is zowel betrokken bij de productie van ROS als bij de biosynthese van glutathion (GSH), een belangrijk antioxidant tijdens Cd stress. Het doel van deze studie is bijgevolg de rol van ethyleenproductie en -signalisatie te ontrafelen in *Arabidopsis thaliana* planten die oxidatieve stress ervaren als gevolg van blootstelling aan sublethale Cd concentraties (5 en 10 µM).

Verschillende studies bestudeerden reeds het effect van Cd blootstelling op de biosynthese van ethyleen in verschillende plantensoorten zonder zich te concentreren op de onderliggende moleculaire mechanismen. Bijgevolg werden in een eerste fase de moleculaire mechanismen bestudeerd die verantwoordelijk zijn voor de stijging in ethyleenproductie na een korte termijn (24 en 72 u) blootstelling aan Cd in wild-type (WT) *A. thaliana* planten **(Hoofdstuk 3)**. Een transiënte toename in transcriptie van verschillende ethyleengevoelige genen gepaard met verhoogde concentraties 1-aminocyclopropaan-1-carboxylzuur

(ACC), een voorloper van ethyleen, ondersteunden deze bevindingen. De productie van ACC door het enzym ACC synthase (ACS) vormt de snelheidsbepalende stap van de ethyleenbiosynthese. De transcripten van *ACS2* en *ACS6*, 2 ACS isovormen, kwamen het meest voor na Cd blootstelling, wat wijst op hun belang tijdens ethyleenproductie veroorzaakt door Cd. Dit werd bevestigd in een studie met *acs2-1acs6-1* dubbele knock-out (KO) mutanten. Cadmium-blootgestelde mutanten vertoonden een verlaagde ethyleenproductie in vergelijking met WT planten, resulterend in een verminderde en vertraagde toename in expressie van de ethyleengevoelige genen.

De relatie tussen deze transiënte ethyleenrespons en Cd-geïnduceerde oxidatieve stress werd vervolgens bestudeerd door verscheidene stressresponsen in wortel en blad van WT *A. thaliana* planten te vergelijken met deze in de *acs2-1acs6-1* mutanten **(Hoofdstuk 4)**. Na korte termijn blootstelling aan 5 µM Cd werd er in de blaadjes van de mutanten een afname in de transcriptie van pro-oxidatieve genen en oxidatieve stress merker genen waargenomen alsook een daling in GSH concentratie. Dit wijst op verminderde oxidatieve stress in de mutanten vergeleken met WT planten. Bijgevolg vertoonden de blaadjes van de *acs2-1acs6-1* mutanten een hoger versgewicht na 72 uur blootstelling aan 5 µM Cd. Blootstelling aan 10 µM Cd daarentegen bleek te intens voor zowel WT planten als de mutanten waardoor de meeste verschillen tussen beide genotypes verdwenen. We veronderstellen dat blootstelling aan hoge concentraties Cd (10 µM) verschillende signalisatiesystemen activeert, bv. oxylipines en jasmonaten, waardoor het gebrek aan ethyleenproductie en -signalisatie in de mutant omzeild kan worden. Bovendien wordt de ontwikkeling en voortplanting van zowel WT planten als mutanten op een gelijkaardige manier verhinderd na blootstelling aan Cd gedurende lange termijn. Dit wijst erop dat ethyleenbiosynthese een belangrijke rol speelt bij de vroege inductie van oxidatieve stress veroorzaakt door blootstelling aan 5 µM Cd.

Gebaseerd op deze bevindingen werd de experimentele resolutie verhoogd om de rol van ethyleensignalisatie in deze vroege Cd-geïnduceerde oxidatieve stress verder te bestuderen **(Hoofdstuk 5)**. Hier werd de respons van WT *A. thaliana* planten in de blaadjes na korte termijn blootstelling aan 5 µM Cd vergeleken met deze in verscheidene mutanten met een verstoorde ethyleen

signaaltransductie: *etr1-1* (*ethylene resistant 1-1*; receptor), *ein2-1* (*ethylene insensitive 2-1*; signalisatie) en *ein3-1* (transcriptiefactor). In de *etr1-1* en *ein2-1* mutanten werd een afwijkende verstoring van de cellulaire redoxbalans door Cd waargenomen in vergelijking met het WT, wat resulteerde in een verminderde groei-inhibitie in deze mutanten. Zowel *etr1-1* als *ein2-1* mutanten vertoonden een vertraagde respons van het GSH metabolisme, dit zowel op niveau van GSH concentratie als transcriptie van genen betrokken in de biosynthese van GSH. Bovendien werd een verlaagde expressie van oxidatieve stress merker genen gemeten in de *ein2-1* mutanten vergeleken met het WT, wat aantoont dat ook ethyleensignalisatie betrokken is in de vroege respons op Cd stress.

Samengevat tonen onze resultaten aan dat zowel ethyleenbiosynthese als -signalisatie betrokken zijn in de regulatie van de vroege Cd-geïnduceerde oxidatieve stress in *A. thaliana* blaadjes blootgesteld aan matige sublethale (5 µM) Cd concentraties.

List of abbreviations

2-OG	2-oxoglutarate
2-VP	2-vinylpyridine
ABC transporter	ATP-binding cassette transporter
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
ACO	ACC oxidase
Al	aluminium
ANOVA	analysis of variance
AP2	apetala2
APX	ascorbate peroxidase
AsA	ascorbate
ATP	adenosine triphosphate
C_2H_4	ethylene
Ca	calcium
CAT	catalase
Cd	cadmium
$CdSO_4$	cadmium sulfate
CDPK	calcium-dependent protein kinase
CTR	constitutive triple response
Cu	copper
DHA	dehydroascorbate
DHAR	DHA reductase
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DW	dry weight
EBF	EIN3 binding F-Box
EIN	ethylene insensitive
EIL	EIN3-Like
ER	endoplasmic reticulum
EREBP	ethylene-responsive element binding protein
ERF	ethylene response factor
ERS	ethylene response sensor

ETC	electron transport chain
ETP	EIN2 targeting protein
ETR	ethylene resistant
Fe	iron
FW	fresh weight
GACC	γ-L-glutamyl-ACC
GR	glutathione reductase
GSH	glutathione (reduced)
GSH1	γ-glutamylcysteine synthetase
GSH2	glutathione synthetase
GSSG	glutathione (oxidised)
H_2O	water
H_2O_2	hydrogen peroxide
Hg	mercury
HMW	high molecular weight
HMA	heavy metal ATPase
KO	knockout
Li	lithium
LMW	low molecular weight
LOX	lipoxygenase
MACC	malonyl-ACC
MDHA	monodehydroascorbate
MDHAR	MDHA reductase
Mg	magnesium
Mn	manganese
MPK	mitogen-activated protein kinase
MTA	methylthioadenosine
Ni	nickel
NRAMP	natural resistance-associated macrophage protein
O_2	atmospheric oxygen
$^1O_2$	singlet oxygen
$^3O_2$	molecular oxygen
$O_2^{\circ-}$	superoxide radical
$^{\circ}OH$	hydroxyl radical

OXI	oxidative signal-inducible
PAR	photosynthetic active radiation
Pb	lead
PC	phytochelatin
PCD	programmed cell death
PCR	polymerase chain reaction
PCS	phytochelatin synthase
PFB	pentafluoro-benzyl
PQ	paraquat
PUFA	polyunsaturated fatty acid
RAN	responsive to antagonist
RBOH	respiratory burst oxidase homologue
ROS	reactive oxygen species
RTE	reversion to ethylene sensitivity
SAM	S-adenosyl-methionine
SOD	superoxide dismutase
VAP	vertical agar plate
VSP	vegetative storage protein
WT	wild type
XRN	exoribonuclease
Zn	zinc

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Chapter 1

Introduction

1.1 Cadmium pollution

The non-essential toxic metal cadmium (Cd) is naturally present in all soils as a divalent cation (Cd^{2+}). It most commonly occurs in zinc (Zn) ores, although a few specific Cd minerals exist in the environment, such as greenockite (CdS) and otavite ($CdCO_3$) (Nriagu and Pacyna, 1988; Smolders and Mertens, 2013). The natural emission of Cd in the environment, for example as a consequence of the weathering of sedimentary rocks, has been exceeded by anthropogenic emission long ago (Clemens, 2006). Since the beginning of the industrial revolution in the 18th century, the pyrometallurgical industry had been focussing on the extraction of zinc and lead (Pb). In Belgium, several Zn smelters were build in the Campine region. The ores used for Zn extraction also contained high concentrations of Cd, that were released it into the environment and eventually accumulated in soils in a vast area around the smelters (Colpaert et al., 2004; Krznaric et al., 2010). Since metal ions are not degradable, their concentrations in the environment remained high (Clemens et al., 2013). Nowadays, the consequences of this historic pollution can still be observed in the Campine region as patches of land lacking most forms of vegetation. In the beginning of the 20th century, the Cd released during the pyrometallurgical processes was collected and used in nickel(Ni)-cadmium batteries, as coating on steel or as stabilisers in plastics (Herbette et al., 2006). Later on, the metal industries replaced these pyrometallurgical processes by more environment-friendly electrochemical processes, strongly decreasing the Cd emission in the European Union. However, this reduction has been mostly negated by the expansion of industrial activities (e.g. mining, burning of fossil fuels) in Asia (Clemens et al., 2013).

The main source of Cd-input in agricultural soils, next to atmospheric deposition, is the addition of phosphate fertilizers or sewage sludge (Clemens et al., 2013; Kirkham, 2006; Smolders and Mertens, 2013). Crops or vegetables grown on such contaminated soils can take up and accumulate Cd, representing an important entry route of Cd into the food chain (Dalcorso et al., 2010). In most parts of the world, the diet is the primary source of environmental Cd exposure

in non-smoking humans. Tobacco smoking, intake of contaminated water, inhalation of polluted air or occupational exposure due to industrial activities represent other potential human exposure pathways. Once absorbed, Cd is mainly stored in the kidneys and liver, with a half-life in the human body of 10-30 years. Therefore, Cd is considered as nephro- and hepatotoxic but it also causes osteoporosis and is considered a class 1 human carcinogen (Gallego et al., 2012; Järup and Akesson, 2009; Nawrot et al., 2010).

1.2 Cadmium responses in plants

The internal Cd concentration of plants differs between species when grown on similar contaminated soils. Cereals, leafy vegetables and root vegetables tend to accumulate more Cd than other food from plants (Järup and Akesson, 2009). Since plant-derived food has a large impact on the human dietary Cd intake, a reorientation from agricultural to non-food crops is occurring in different Cd-contaminated areas (Clemens et al., 2013; Gallego et al., 2012). The selection of these crops is based on their metal resistance as well as accumulation capacity, aiming to stabilise and/or clean these soils in a process termed phytoremediation. Consequently, it is necessary to elucidate the Cd-induced effects in plants, an important factor in optimising the phytoremediation process, eventually alleviating the Cd-associated threats for the public health (Vangronsveld et al., 2009; Weyens et al., 2012).

1.2.1 Uptake and transport of Cd

Because Cd is highly water soluble, it is highly bioavailable in most soil conditions as well as in water and primarily enters plants via the root system. Only a fraction of the internal Cd concentration in plants is airborne (Clemens, 2006). The uptake of Cd in plants depends on the plant species as well as the availability and concentration of Cd, strongly influenced by different soil parameters such as pH, organic matter and the rhizosphere. The first barrier affecting Cd entrance into the cell is the cell wall, which because of its negative charge has a significant Cd binding capacity (DalCorso et al., 2008; Gallego et al., 2012; Van Belleghem et al., 2007). To enter the cell, non-essential metal ions such as Cd utilise the same transporters used for essential nutrients, since

no specific Cd-uptake mechanisms exist (Fig. 1.1). In particular, Cd competes with the uptake of Ca, Fe, Mg, Cu and Zn. The negative membrane potential as well as the presence of intracellular metal binding sites provides a strong driving force for the uptake of cations (Clemens, 2006; Mendoza-cózatl et al., 2012; Roth et al., 2006).

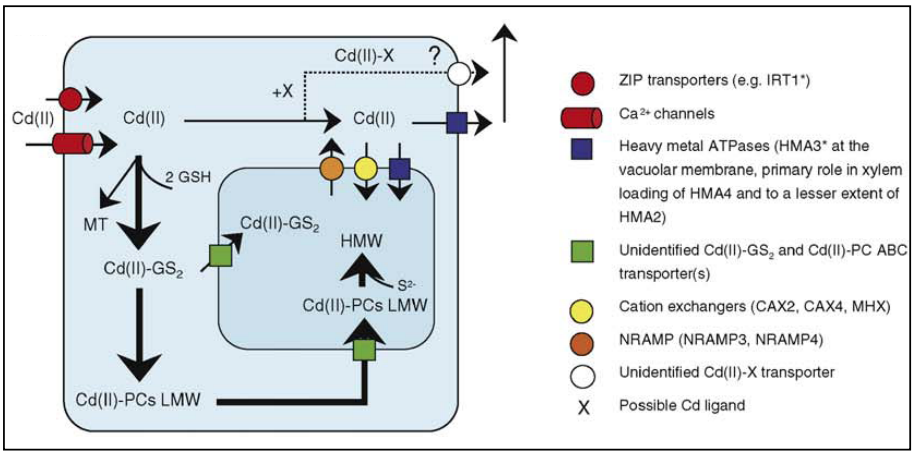

Figure 1.1. *A schematic overview of the processes involved in Cd uptake, vacuolar sequestration and translocation in roots (adapted from: Verbruggen et al., 2009). Only the vacuole is shown inside the cell. Line thickness is related to flux rate. Cd^{2+} ions are taken up by Fe^{2+} and Zn^{2+} ZIP transporters or by Ca^{2+} transporters/channels. The main detoxification pathway of Cd in roots relies on PC complexation and vacuolar transport of Cd-PCs complexes of low molecular weight (LMW). In the vacuole, high molecular weight (HMW) complexes are formed. Cd^{2+} ions can also be transported in the vacuole by different transporters (Heavy Metal ATPase 3, cation exchangers) or bound to glutathione. A part of the vacuolar Cd^{2+} is effluxed back into the cytosol by Natural Resistance-Associated Macrophage Protein (NRAMP) transporters. Ionic Cd can be loaded into the xylem by HMA2 and mainly HMA4.*

Inside the cell, these non-essential toxic metal ions can disturb the metabolism by for example interfering with the function of proteins and enzymes. To avoid this, plants developed different mechanisms including the chelation and sequestration of free metal ions by low molecular weight (LMW) molecules. The favoured ligands of Cd are thiols, present in phytochelatins (PCs) and their precursor glutathione (GSH). They can form complexes with Cd that are

transported into vacuoles, removing it from the cytosol via ATP-binding cassette transporters (ABC transporters). Unbound Cd ions can also enter the vacuole by the activity of Heavy Metal ATPase 3 (HMA3) or cation exchangers, and even be released back into the cytosol via Natural Resistance-Associated Macrophage Protein (NRAMP) activity (Fig. 1.1). Membrane transporters, loading Cd into the xylem, mediate transport of Cd to the aerial plant parts (Fig. 1.1). In leaf cells, the molecular mechanisms of Cd chelation and sequestration are similar to those employed by root cells. Furthermore, phloem-loading processes play an important role in delivering nutrients to developing seed, potentially accumulating toxic metals (Clemens, 2006; Mendoza-cózatl et al., 2012; Seth et al., 2012; Verbruggen et al., 2009).

1.2.2 Cadmium phytotoxicity

When the internal Cd concentration exceeds the capacity of the detoxification mechanisms, it becomes phytotoxic (Lin and Aarts, 2012). Evident symptoms of Cd phytotoxicity are stunted growth and chlorosis, caused by an imbalance in water and nutrient uptake and a decreased photosynthetic activity (Dalcorso et al., 2010).

At a cellular or molecular level, Cd toxicity can result from interactions with thiol-, histidyl- and carboxyl-groups in proteins, disrupting their structure or interfering with their function (Hall, 2002; Sharma and Dietz, 2009). Another important mechanism is due to the similarity between Cd and essential ions, for example situated in the active site of enzymes and signalling components, deregulating their function (DalCorso et al., 2008; Roth et al., 2006). Finally, although Cd is not redox-active, an important cellular response to increased internal Cd concentrations is the generation of reactive oxygen species (ROS), disturbing the redox balance and resulting in an oxidative challenge (Cuypers et al., 2009; Sharma and Dietz, 2009).

1.3 Cadmium-induced oxidative challenge

Under normal physiological conditions, ROS are unavoidably produced as by-products during basic cellular processes. In plants, the electron transfer activities of chloroplasts and mitochondria, and the oxidative metabolism of the

peroxisomes constitute the predominant source of ROS (Kucera et al., 2008; Noctor et al., 2007; Sharma and Dietz, 2009). The activation of oxygen to ROS can occur in two different ways. First, molecular oxygen in its ground state ($^3O_2$) can be transformed into singlet oxygen ($^1O_2$) by the input of energy. Furthermore, the stepwise univalent reduction of O_2 can generate superoxide radicals ($O_2°^-$), hydrogen peroxide (H_2O_2) or hydroxyl radicals (°OH) (Halliwell, 2006). The generation of ROS is maintained at non-toxic levels in a delicate balancing act by the antioxidative defence network (Baxter et al., 2014; Mittler et al., 2004). The Cd-induced oxidative challenge disturbs this equilibrium, causing enhanced ROS levels via the activation of pro-oxidative mechanisms or the inhibition of antioxidative defence mechanisms (Cuypers et al., 2012).

1.3.1 Indirect Cd-induced ROS production

Redox-active transition metals such as Cu and Fe can directly generate ROS via Fenton and Haber-Weiss reaction (Fig. 1.2). The formation of hydroxyl radicals, one of the most reactive oxygen species known, can initiate radical chain reactions, irreversibly damaging cellular components. These radicals are for example involved in the non-enzymatic peroxidation of lipids, leading to membrane damage. Since Cd is a non redox-active metal, it is unable to directly generate ROS through these reactions. However, Cd can replace essential ions such as Fe and Cu from their functional site in proteins, thereby generating free redox-active metals capable of initiating these reactions (Fig. 1.2) (Cuypers et al., 2010; Cuypers et al., 2012; Mithöfer et al., 2004).

	$\mathbf{M^{(n)}} + O_2°^- \longrightarrow \mathbf{M^{(n-1)}} + O_2$
Fenton	$\mathbf{M^{(n-1)}} + H_2O_2 \longrightarrow \mathbf{M^{(n)}} + °OH + OH^-$
Haber-Weiss	$O_2°^- + H_2O_2 \longrightarrow O_2 + °OH + OH^-$

Figure 1.2. *Fenton and Haber-Weiss reactions. Oxidised transition metal ($M^{(n)}$, e.g. Fe^{3+}, Cu^{2+}), reduced transition metal ($M^{(n-1)}$, e.g. Fe^{2+}, Cu^{+}), oxygen (O_2), superoxide ($O_2°^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (°OH) and hydroxide ion (OH^-).*

Furthermore, Schützendübel and Polle (2002) observed a Cd-induced depletion of GSH. Since GSH and their oligomers PC, are a preferred ligand for Cd (Fig. 1.1), and GSH is also involved in the detoxification of pro-oxidants, this resulted in the intracellular accumulation of H_2O_2 (Jozefczak et al., 2014; Schützendübel and Polle, 2002). The inhibition of the antioxidative enzymes, glutathione reductase (GR), ascorbate peroxidase (APX) and catalase (CAT) by Cd additionally disturbs the functioning of the antioxidative defence mechanism (Schützendübel and Polle, 2002).

1.3.2 Enzymatic Cd-induced ROS production

Plasma-membrane-localised NADPH oxidases generate apoplastic $O_2^{\bullet -}$ by transferring electrons from cytosolic NADPH to extracellular molecular oxygen (Chmielowska-Bąk et al., 2014). Due to their homology with the mammalian respiratory burst oxidase $gp91^{phox}$, these enzymes are also called RBOH (respiratory burst oxidase homologues) (Torres et al., 1998). Recent studies have revealed that RBOHs are involved in a multitude of signalling pathways, including defence reactions and acclimation to abiotic stresses (Baxter et al., 2014). In response to stress stimuli such as Cd, plants react by increasing the flux of Ca into the cytosol. The binding of Ca to the EF-hand motifs of NADPH oxidases stimulates calcium-dependent protein kinases (CDPKs) that phosphorylate their N-terminal domain, hereby facilitating their activation. Moreover, Cd ions can also directly stimulate the activation of NADPH oxidases by mimicking Ca ions (Cuypers et al., 2012). Finally, Remans et al. (2010) observed increased expression of different NADPH oxidase genes after Cd exposure in *A. thaliana*.

Lipoxygenases are also often induced during stress conditions. They catalyse the dioxygenation of polyunsaturated fatty acids (PUFAs) producing hydroperoxy fatty acids, potentially evoking lipid peroxidation. Subsequent enzymatic modifications generate different oxylipins, some of them belonging to the jasmonate family, also involved in signalling following (a)biotic stresses (Montillet et al., 2004). Increased LOX activity was observed in *Arabidopsis thaliana* plants under Cd stress (Tamás et al., 2009). In barley root tips, Cd exposure led to excessive LOX activity, enhancing lipid peroxidation (Skórzyńska-Polit et al., 2006). Furthermore, Remans et al. (2010) also

observed increased transcript levels of several LOX isoforms after Cd-exposure in roots and leaves of *A. thaliana*. Finally, LOX1 was shown to be involved in stress signalling following Cd exposure in *A. thaliana* by Keunen et al. (2013).

1.3.3 Cadmium-induced ROS production: damage versus signalling

The enhanced ROS production during the Cd-induced oxidative challenge can result in cellular damage (Cuypers et al., 2012). Excessive ROS, particularly free °OH produced during Fenton and Haber-Weiss reactions, can react with virtually all biomolecules including lipids, DNA, carbohydrates and proteins, possibly leading to necrosis and cell death (Møller et al., 2007). To avoid this and maintain the cellular redox homeostasis within its physiological limits, plants have developed an antioxidative defence system consisting of enzymatic components, e.g. superoxide dismutase (SOD), CAT and APX, and metabolic components, e.g. ascorbate (AsA) and GSH.

The SODs catalyse the dismutation of $O_2^{\circ -}$ to H_2O_2, which on its turn is scavenged by CAT and APX (Fig. 1.3) (Mittler et al., 2004). The metabolites, AsA and GSH can also scavenge ROS directly or cooperate with enzymes, together constituting the AsA-GSH cycle. This cycle reduces H_2O_2 to H_2O, oxidising AsA. Ascorbate is again reduced enzymatically by monodehydroascorbate reductase (MDHAR), using GSH as an electron donor. Glutathione reductase (GR) reduces the oxidised form of glutathione (GSSG) to GSH in the presence of NAD(P)H (Fig. 1.3) (Bielen et al., 2013; Jozefczak et al., 2012; Sobrino-Plata et al., 2014).

Maintaining controlled levels of ROS is important because they are also known to play a key role in plants as signal transduction molecules, modulating various physiological processes and (a)biotic stress responses. They are small molecules, able to diffuse over short distances (Miller et al., 2010; Mittler et al., 2004). Cadmium-induced ROS generation is known to interact with mitogen activated protein kinase (MAPK) pathways, important signalling modules converting receptor signals to cellular responses. The activation as well as mRNA levels of MPK3 and MPK6 were shown to be induced after Cd exposure in *A. thaliana* (Liu et al., 2010; Opdenakker et al., 2012). Both MPK3 and MPK6 are capable of phosphorylating 1-aminocyclopropane-1-carboxylic acid (ACC) Synthase 2 and 6 (ACS2/6), the rate limiting enzymes of ethylene biosynthesis,

reducing their turnover time and potentially increasing the ethylene production (Han et al., 2010; Joo, Liu, Lueth, & Zhang, 2008; Y. Liu & Zhang, 2004a). Moreover, ethylene is known to mediate ROS production under different stress conditions (Mersmann et al., 2010; Montero-Palmero et al., 2014a).

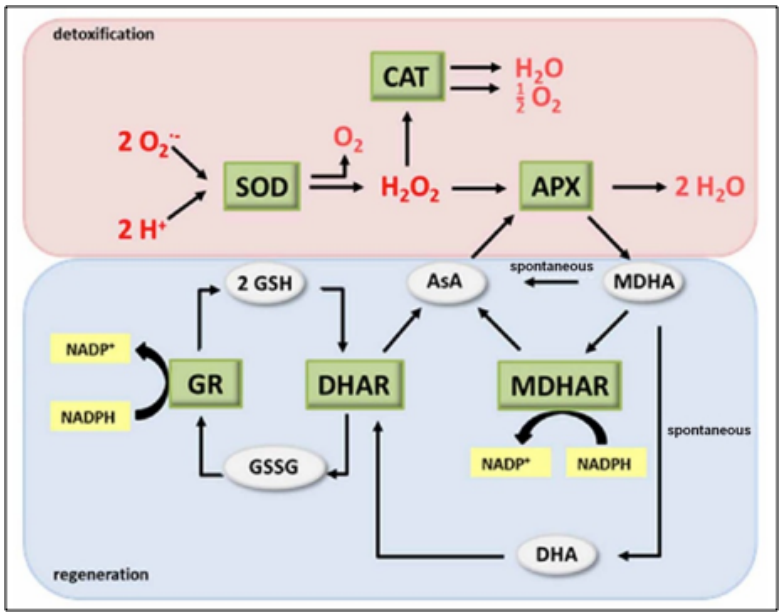

Figure 1.3. *Schematic overview of the antioxidative defence system in plants (adapted from: Groß et al., 2013), see main text for further details. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), ascorbate (AsA), monodehydroascorbate (MDHA), dehydroascorbate (DHA), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), reduced glutathione (GSH), oxidised glutathione (GSSG), glutathione reductase (GR) (See main text for further details).*

1.4 The Cd-induced effects on ethylene biosynthesis and signalling

The gaseous plant hormone ethylene (C_2H_4) was first identified in 1901 by Dimitri Neljubov as the active component in illuminating gas, causing premature senescence, abscission and ripening in nearby vegetation (Neljubov, 1901). Nowadays, it is known that ethylene is produced in all cells during plant development. However, the production rate varies, with the highest rates being associated with meristematic, ripening or stressed tissues. As a gas, ethylene can dissolve in both lipid membranes and the aqueous phase of cells, although 14 times better in lipids. Although ethylene is best known as the ripening hormone, many aspects of the plant's life cycle are influenced by ethylene. These include, seed germination, root initiation, flower development, sex determination and senescence but also responses to biotic and abiotic stresses. Furthermore, ethylene also influences plant growth in both darkness and light. The triple response phenotype, observed in ethylene-treated etiolated *Arabidopsis* seedlings: inhibition of hypocotyl and root elongation, radial swelling of hypocotyl and root cells and exaggerated apical hook, has been used to screen for mutants that are defective in ethylene responses. This was a useful tool in elucidating the ethylene biosynthesis and signalling pathway (Abeles, 1992; Bleecker et al., 1988; Lin et al., 2009; Smalle and Van Der Straeten, 1997; Vandenbussche et al., 2012).

1.4.1 Ethylene biosynthesis

The biosynthesis of ethylene occurs through a relatively simple pathway and was principally elucidated by Yang and co-workers (Fig. 1.4) (De Paepe and Van Der Straeten, 2005; Yang and Hoffman, 1984). The biological precursor of ethylene is methionine, which is converted to S-adenosylmethionine (SAM) by SAM synthetase. SAM is the substrate of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), forming ACC. This is mostly the rate-limiting step in the biosynthesis of ethylene and requires pyridoxal phosphate (PLP) as a cofactor. In addition, ACS also produces 5'-methylthioadenosine (MTA), which is subsequently recycled to methionine in the methionine cycle. ACC is further oxidised by ACC oxidase (ACO) to ethylene, CO_2 and cyanide, which is detoxified

to β-cyanoalanine by β-cyanoalanine synthase to prevent toxicity (Fig. 1.4) (Bleecker and Kende, 2000; De Paepe and Van der Straeten, 2005; Lin et al., 2009; Vandenbussche et al., 2012).

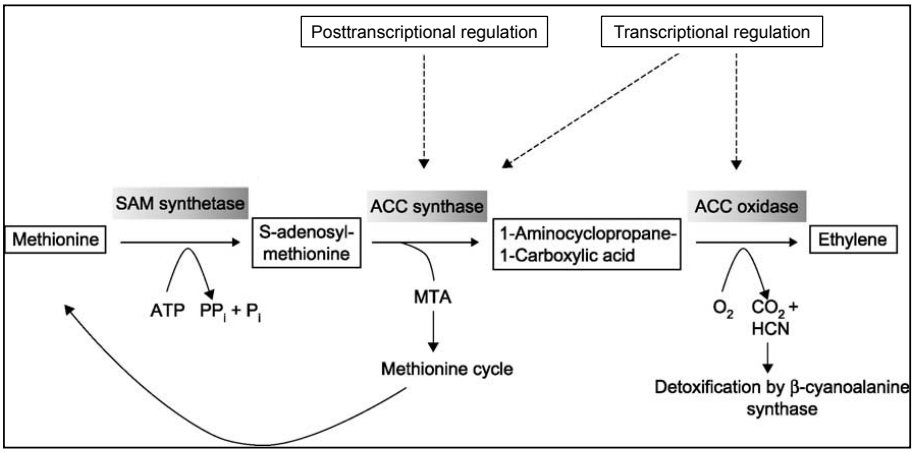

Figure 1.4. *Biosynthetic pathway of ethylene and its regulation (adapted from: De Paepe and Van Der Straeten, 2005). The formation of S-adenosyl-methionine (SAM), using methionine as a substrate, is catalysed by SAM synthetase. The conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) is the rate-limiting step of ethylene biosynthesis. Methylthioadenosine (MTA) is recycled to methionine. ACC oxidase (ACO) catalyses the final step of the ethylene biosynthesis, using ACC and generating ethylene. ACS and ACO can be transcriptionally regulated; furthermore ACS can also be posttranscriptionally regulated.*

In *A. thaliana*, a 12-membered multigene family encodes the different ACS isoforms. Because *ACS3* is a pseudogene and *ACS10* and *ACS12* encode aminotransferases with different functions, only 9 actual *ACS* genes remain. Furthermore, *ACS1* is only active as a heterodimer (Yamagami et al., 2003). These genes are differentially regulated at the transcriptional level by developmental as well as environmental signals in response to internal and external stimuli (e.g. ripening, abscission, wounding, chilling, hormones, etc.). All the isoforms display distinct spatial and temporal expression patterns throughout the plant's life and in response to various stressors. For example, the transcript levels of *ACS8* were reported to be controlled by light and shade as well as the circadian clock (Thain et al., 2004; Vandenbussche et al., 2003). The

expression of *ACS2* and *ACS6* often appears to be regulated by different kinds of stress such as ozone, salinity and hypoxia (Arteca and Arteca, 1999; Peng et al., 2005). In addition, the ACS enzymes have a highly variable carboxylic end, serving as a regulatory domain responsible for post-transcriptional regulations (Tsuchisaka et al., 2009). Based on their C-terminal sequences, they can be divided into three main groups. Type 1 proteins have extended C-termini containing target sites for MPK and probably also CDPK phosphorylation. The type 2 proteins carry only the CDPK target motif, while type 3 proteins possess neither target site (Han et al., 2010; Lin et al., 2009; Y. Liu and Zhang, 2004; Skottke et al., 2011). Finally, functional homo- and heterodimeric interactions among the ACS enzymes exist, increasing their versatility and enhancing their biochemical diversity (Lin et al., 2009; Peng et al., 2005; Tsuchisaka and Theologis, 2004; Yamagami et al., 2003).

The immediate precursor of ethylene, ACC, can be reversibly removed from the biosynthetic pathway of ethylene through conjugation to malonyl-ACC or γ-L-glutamyl-ACC (MACC, GACC) (McDonnell et al., 2009; Plett et al., 2009). The accumulation of conjugated (inactive) ACC could optimise free ACC levels as a substrate for ACO, which catalyses the final step of the ethylene biosynthesis (Fig. 1.4). In conditions of high ethylene production, such as fruit ripening, ACO can also serve as the rate-limiting step in biosynthesis. It is a member of ferrous-dependent non-heme oxygenases, most of which use 2-oxoglutarate (2-OG) as a co-substrate. In *Arabidopsis thaliana*, a five-membered multigene family encodes the different ACO isoforms. The *ACO* genes appear to be expressed in all plant tissues. However, differences in accumulation of specific *ACO* transcripts are observed depending on the physiological processes and environmental conditions (Argueso et al., 2007; De Paepe and Van der Straeten, 2005; Lin et al., 2009; Ruduś et al., 2012).

The Cd-induced effects on the biosynthesis of ethylene have been investigated in several studies (Abeles, 1992). Groppa et al. (2003) reported an increased ethylene production in 4-week-old wheat leaves after 14 h exposure to 1 mM of Cd whereas in sunflower leaves this increased ethylene production was absent. Exposure to 400 μM Cd differently induced ethylene production in various plant parts of *A. thaliana*, including roots, leaves, buds and stalks. The induction was inversely proportional to the age of the plant parts (Arteca and Arteca, 2007).

Rodríguez-Serrano et al. (2009) detected an increased ethylene production in 14-day-old pea plants exposed to 50 µM Cd during 14 days. Masood et al. (2012) reported an increased ethylene production in the leaves of mustard plants after treatment with 200 mg Cd kg^{-1} soil at 30 days after sowing. Recently, Chmielowska-Bąk et al. (2013) also measured an increased ethylene production in the roots of soybean seedlings treated with 10 or 25 mgL^{-1} Cd during 6 to 24 h.

These results indicate that the effect of Cd on the ethylene production is concentration specific, but also species as well as plant part specific. The mechanistic basis of these effects however remains unclear.

1.4.2 Ethylene signal transduction

The ethylene signalling pathway starts with the perception of ethylene by a family of membrane-bound, predominantly endoplasmic reticulum (ER)-located receptors (Fig. 1.5). Although this is not a typical site for receptor-ligand binding, the gaseous nature of ethylene allows it to diffuse freely to the ER through the aqueous and lipid environments of the cell. In *A. thaliana*, a five-membered family of negatively regulating ethylene receptors exists: ETHYLENE RESISTANT 1 & 2 (ETR1 & ETR2), ETHYLENE RESPONSE SENSOR 1 & 2 (ERS1 & ERS2) and ETHYLENE INSENSITIVE 4 (EIN4). They all share a modular structure composed of an N-terminal transmembrane ethylene binding domain, a domain involved in the protein-protein interactions between different receptor types, and a C-terminal domain necessary for the interaction with downstream components. The different C-termini of the receptors show sequence homology to the bacterial two-component system histidine kinases. Based on these similarities, the five receptors can be classified into two subfamilies. ETR1 and ERS1, belonging to subfamily 1, possess histidine autokinase activity as in the two-component system. The subfamily 2 members, ETR2, ERS2 and EIN4 are more diverged, possessing serine/threonine kinase activity and an additional N-terminal hydrophobic domain.

The N-terminal ethylene-binding domain of the receptors uses Cu as a cofactor, provided by the RESPONSIVE TO ANTAGONIST 1 (RAN1) copper transporter. Signalling from ETR1 is promoted by interacting with another ER-localised protein REVERSION TO ETHYLENE SENSITIVITY 1 (RTE1). All receptors form

homo- and heterodimers, which reflect the functional unit of the receptors. Higher order associations can also occur among homodimers. Furthermore, they are largely redundant in the control of ethylene responses, although some functional specificity exists (Bisson and Groth, 2010; Hua and Meyerowitz, 1998; Merchante et al., 2013; Qiao et al., 2012; Schaller and Bleecker, 1995).

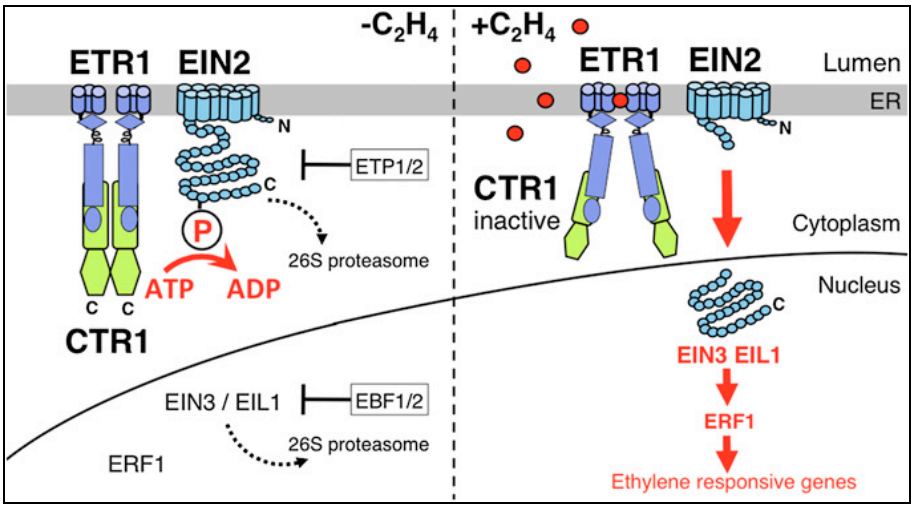

Figure 1.5. *Signal transduction pathway of ethylene (adapted from: Ju et al., 2012). In the absence of ethylene (left), the receptors (e.g. ETR1) at the ER membrane activate CTR1, a dimer that phosphorylates the C-terminal domain of EIN2, preventing its nuclear localisation. EIN2 is targeted for 26S proteasomal degradation by the F-box proteins ETP1/2. EIN3/EIL1 are also targeted for degradation by F-box proteins EBF1/2. In the presence of ethylene (right), the receptors are inactivated, hereby inactivating CTR1. The absence of phosphorylation on EIN2 results in the EIN2 C-terminus being cleaved and localised to the nucleus. This activates the downstream cascade, EIN3/EIL1, ERF1 and the ethylene responsive genes.*

In the absence of ethylene the receptors activate CONSTITUTIVE ETHYLENE RESPONSE 1 (CTR1), which functions as a negative regulator of the ethylene signalling pathway (Fig. 1.5) (Kieber et al., 1993). CTR1 is a Ser/Thr protein kinase that forms homodimers when activated. It is located at the ER membrane because of the association of the N-terminal regulatory domain with the receptors. The kinase domain is located in the C-terminus and is necessary for the downstream signalling (Gao et al., 2003; Huang et al., 2003; Mayerhofer et

al., 2012). Due to its sequence similarities with Raf protein kinases, CTR1 has long been presumed to function as a mitogen-activated protein kinase kinase kinase (MAPKKK). However, to date, no conclusive CTR1-targeted MAPKKs or MAPKs have been identified (Ju et al., 2012; Merchante et al., 2013; Zhao and Guo, 2011).

Downstream of CTR1, is ETHYLENE INSENSITIVE 2 (EIN2), an essential positive regulator of ethylene signalling (Fig. 1.5). Its central role in ethylene signalling is further supported by the fact that *EIN2* is the only gene of all components in the ethylene pathway whose loss-of-function mutation leads to complete ethylene insensitivity (Alonso et al., 1999). The hydrophobic N-terminal domain of the EIN2 protein consists of a predicted 12-fold transmembrane region (helices), while the hydrophilic C-terminus harbours a conserved nuclear localisation sequence (Wen et al., 2012). The N-terminal domain has a sequence similarity to NRAMP metal ion transporters, although no transport activity for EIN2 has been shown (Merchante et al., 2013). The EIN2 protein is localised in the ER membrane, were it physically interacts with the kinase domain of the ethylene receptors (Bisson et al., 2009; Bisson and Groth, 2010). It was recently shown that in the presence of ethylene, EIN2 lacks phosphorylation at multiple serine and threonine residues, while in the absence of ethylene CTR1 phosphorylates the C-terminal end of EIN2 through a physical interaction, preventing it from downstream signalling. Upon ethylene treatment EIN2 accumulates, and once inside the nucleus, the C-terminal end of EIN2 leads to the activation of the ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) dependent transcriptional cascade (Ju et al., 2012; Qiao et al., 2012).

EIN3 and its homologs (EILs) are short-lived proteins accumulating in the nucleus upon ethylene treatment, and positively regulating the ethylene-signalling pathway (Fig. 1.5). In *Arabidopsis thaliana*, there are 5 EIL proteins (EIL1-EIL5), of which EIL1 is closest related to EIN3. The cellular roles of the remaining EIL2-5 members of the family remain unclear, although EIL2 could also play a minor role in the ethylene perception. EIN3 and EIL1 are the two master transcription factors generating the primary output of ethylene responses (Fig. 1.5) (An et al., 2010; Binder et al., 2007; Chao et al., 1997; Merchante et al., 2013; Solano et al., 1998; Yoo et al., 2009).

The protein turnover of EIN2 and EIN3/EIL1 is regulated by 26S proteasome-mediated degradation following ubiquitination by E3 ligases containing different F-box proteins. EIN2 TARGETING PROTEIN 1 & 2 (ETP1 & 2) are F-box proteins that negatively regulate EIN2 protein levels through physical interactions. Protein levels of ETP1/2 are downregulated by ethylene, hereby accumulating EIN2 (Qiao et al., 2009). The levels of EIN3 and EIL1 are regulated by the F-box proteins EIN3 BINDING F-BOX PROTEIN 1 & 2 (EBF1/2), which are downregulated by ethylene in an EIN2-dependent manner. It has been shown that EBF1 and EBF2 have different roles in regulating EIN3 stability. Whereas EBF1 exerts its effect primarily in the absence of ethylene and during the initial phase of the response, EBF2 plays a more prominent role during the later stages of the response and the resumption of growth following ethylene removal (An et al., 2010; Binder et al., 2007). The removal of EBF proteins leads to the accumulation of EIN3. A direct negative feedback regulation between EBF2 and EIN3 exists since *EBF2*, and not *EBF1*, is transcriptionally induced by EIN3. Hence, the balance between the ethylene dependent transcription of *EBF2* and protein removal of EBF1 and EBF2 modulates the plant's responsiveness to ethylene through EIN3/EIL1 stability (De Paepe et al., 2005; Konishi and Yanagisawa, 2008; Merchante et al., 2013; Vandenbussche et al., 2012). Finally, the EXORIBONUCLEASE4/ETHYLENE INSENSITIVE 5 (XRN4/EIN5) downregulates the *EBF1* and *EBF2* mRNA levels by an unknown mechanism responsive to ethylene (Olmedo et al., 2006; Potuschak et al., 2006).

Both EIN3 and EIL1 induce/regulate the expression of ethylene responsive factors (ERFs), such as ERF1, which function as ethylene responsive element binding proteins (EREBPs) that stimulate the transcription of target genes (Fig. 1.5) (Solano et al., 1998; Vandenbussche et al., 2012; Zhu et al., 2011). The expression of different ERF proteins (e.g. ERF1 and ERF2), belonging to the APETALA2 (AP2)/EREBP family, was shown to be upregulated in roots of *Arabidopsis thaliana* after 2 h exposure to 50 µM Cd, controlling the expression of other stress-related genes (Dalcorso et al., 2010; Weber et al., 2006). This AP2/EREBP transcription factor family is known to mediate during hormone as well as redox signalling processes in the context of abiotic stresses (Dietz et al., 2010; Montero-Palmero et al., 2014b).

1.5 The Cd-induced oxidative challenge and its relation to ethylene

The link between oxidative stress and phytohormones has been extensively investigated. Their interactions include the regulation of various developmental processes such as seed germination, growth and programmed cell death (Diaz-Vivancos et al., 2013; Mittler et al., 2011; Overmyer et al., 2003). Increasing evidence also suggests a major role for plant hormones and their interaction with cellular redox signalling in order to control defence responses to environmental stresses (Bartoli et al., 2013; Baxter et al., 2014). In particular, the stress hormone ethylene is known to mediate hormone and redox signalling processes during (a)biotic stress (Chmielowska-Bąk et al., 2014; Dietz et al., 2010; Montero-Palmero et al., 2014b).

Different studies have reported a role for ethylene in the stress-induced oxidative burst. Inhibition of ethylene production or perception by 2-aminoethoxyvinylglycine or silver thiosulphate respectively, blocked the H_2O_2 production induced by camptothecin, a cell death inducer, in tomato suspension cells (de Jong et al., 2002). Moreover, a decreased H_2O_2 production was measured in paraquat- (PQ) and aluminium- (Al) treated ethylene insensitive *ein2-1 A. thaliana* mutants as compared to wild-type (WT) plants (Cao et al., 2006; Zhang et al., 2014). In addition, Mersmann et al. (2010) observed a diminished ROS generation after flagellin FLS22 treatment in the ethylene insensitive *etr1* and *ein2* mutant *A. thaliana* plants. This was related to a decreased activation of the RBOH isoform D (RBOHD) through an impaired ethylene signalling. Finally, treatment with the ethylene receptor inhibitor 1-methylcyclopropene abolished the increase in NADPH oxidase activity after exposure to the toxic metal Hg in alfalfa roots (Montero-Palmero et al., 2014a).

Stress-mediated ethylene signalling is also known to affect GSH biosynthesis. Yoshida et al. (2009) observed increased GSH levels in ozone-exposed WT *A. thaliana* plants after 6 h, which was absent in *ein2* mutants. Both expression and activity of the GSH synthesising enzymes GSH1 and GSH2 were significantly lower in the *ein2* mutants as compared to the WT plants, suggesting that ethylene increases *de novo* GSH biosynthesis. This was supported by Cao et al. (2009), who also measured a reduced expression of *GSH1* in lead (Pb)-exposed *ein2-1 A. thaliana* mutants, resulting in decreased GSH levels.

The link between ethylene and the Cd-induced oxidative challenge has only been scarcely investigated up to now. Masood et al. (2012) observed that treatment with the ethylene source ethephon led to increased GSH levels in Cd-exposed mustard plants. Furthermore, a decreased lipid peroxidation in roots, leaves and fruits was observed in the ethylene insensitive *Never ripe* (*Nr*) tomato mutants as compared to WT plants after Cd exposure (Gratão et al., 2012). Finally, Yakimova et al. (2006) showed that ethylene signalling is an important component during Cd-induced cell death in tomato suspension cells.

In conclusion, it is known that (1) Cd exposure induces an oxidative challenge in plants and (2) an interaction between oxidative stress and ethylene exists. Moreover, stress-induced ethylene production followed by regular ethylene signalling is known to inhibit plant development and accelerate senescence and abscission processes. Therefore, a better understanding of the effects of Cd on the ethylene biosynthesis and signalling pathways is crucial to unravel the link between ethylene and the Cd-induced oxidative challenge. This can further improve our knowledge on plant Cd resistance, potentially contributing to the development and/or selection of crops to be used in the phytoremediation of Cd-contaminated soils.

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Chapter 2

Objectives

Toxic metal contamination in soils caused by natural but primarily anthropogenic sources, such as mining and agricultural or industrial activities, is a severe problem worldwide. In Belgium and the Netherlands, an area of approximately 700 km^2 (Campine region) is contaminated with cadmium (Cd) due to historical pollution by zinc (Zn) smelters (Lin and Aarts, 2012). The bioaccumulation of Cd in plants ultimately leads to the introduction of Cd into the food chain, eliciting threats to the public health even when present in trace amounts (Clemens et al., 2013).

The highly phytotoxic non-essential element Cd disturbs several physiological processes in plants, inducing stunted growth and reduced photosynthetic activity (Dalcorso et al., 2010). At the cellular level, its phytotoxicity is highly associated with the generation of reactive oxygen species (ROS), although Cd is not redox-active, resulting in a Cd-induced oxidative challenge. Excessive ROS can damage various plant biomolecules including lipids, DNA and proteins. However, controlled levels of ROS, maintained by the antioxidative defence system, are important since they act as signal transduction molecules, potentially involved in plant acclimation to abiotic stress such as Cd exposure (Cuypers et al., 2012).

Phytohormones are also important signalling molecules, integrating many aspects of growth and developmental programs during the plant's life cycle, and regulating responses to environmental stimuli. The gaseous plant hormone ethylene is often considered as the 'stress hormone', modulating defence responses induced by a variety of stress signals (De Paepe and Van Der Straeten, 2005). Although the effect of Cd on the ethylene biosynthesis has already been investigated, the molecular mechanisms behind this remain unclear (reviewed in Chmielowska-Bak et al., 2014). Furthermore, increasing evidence suggests a link between ethylene and redox signalling processes in the control of defence responses to (a)biotic stresses. Stress-mediated ethylene has been reported to induce the oxidative burst and glutathione (GSH) biosynthesis (Montero-Palmero et al., 2014; Yoshida et al., 2009). Nevertheless, the link between ethylene production, signalling and the Cd-induced oxidative challenge has only been scarcely investigated up to now.

In this study it is hypothesised that the oxidative challenge induced by sublethal environmentally realistic Cd concentrations (5 and 10 µM Cd) in *Arabidopsis thaliana* plants relies on both ethylene biosynthesis as well as signal transduction.

This hypothesis was investigated based on three research objectives:

1. The first objective was to **unravel the molecular mechanisms of Cd-enhanced ethylene biosynthesis (Chapter 3)**. To investigate this research objective, the effect of Cd on the biosynthesis of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, and ethylene production was analysed in wild-type (WT) *A. thaliana* plants after Cd exposure. In addition, the expression of genes involved in ACC and ethylene biosynthesis was investigated in roots and leaves of WT plants after 24 & 72 h Cd exposure.
   The conversion of s-adenosylmethionine (SAM) to ACC by ACC synthase (ACS) is the rate-limiting step in ethylene biosynthesis, and *ACS2* and *ACS6* often appear to regulate the production of stress-ethylene (Skottke et al., 2011). Therefore, ethylene production was analysed in *acs2-1acs6-1* double knockout (KO)-mutants upon Cd exposure. Moreover, Cd-induced effects on the fresh weight and the expression of ethylene responsive genes were investigated and compared between both WT and *acs2-1acs6-1* double KO-mutant plants.

2. From the first part, it became clear that ethylene is a crucial mediator in the early response to Cd stress (Schellingen *et al.* 2014). Therefore, the second objective was to **investigate the short-term influence of the transient increase in ethylene production on the Cd-induced oxidative challenge and plant growth as well as the consequent long-term influence on plant acclimation (Chapter 4)**.
   To compare oxidative stress levels in the roots and leaves of both WT and *acs2-1acs6-1* double KO-mutant *A. thaliana* plants, the Cd-induced effects on transcript levels of pro-oxidative and oxidative stress hallmark genes (Gadjev et al., 2006) were measured.

Glutathione is an important antioxidant involved in the plant's response to Cd stress. It plays a role in Cd chelation and in the control of the oxidative challenge (Jozefczak et al., 2014). Furthermore, ethylene is known to affect GSH biosynthesis (Yoshida et al., 2009). Consequently, the expression of the genes encoding the GSH synthesising and recycling enzymes as well as the content of GSH after short-term Cd exposure in roots and leaves of both genotypes were investigated.
Finally, short- and long-term effects of Cd exposure on the growth of WT and *acs2-1acs6-1* mutant plants as well as long-term effects on survival and reproduction capacity of both genotypes were analysed.

3. In the third objective we increased our experimental resolution to **study the link between the oxidative challenge caused by short-term exposure to moderate Cd concentrations (5 µM) and ethylene signalling in *A. thaliana* leaves (Chapter 5)**. The importance of ethylene during these experimental conditions was proven by the results of the second objective.
To investigate the existence of a link between the Cd-induced oxidative challenge and ethylene signalling, different mutant plants with an impaired ethylene signal transduction pathway, *ethylene resistant* (*etr*)*1-1* (receptor), *ethylene insensitive* (*ein*)*2-1* (signal transducer) and *ein3-1* (transcription factor) were used.
In accordance with the second objective, the Cd-induced effects on (1) plant growth, (2) the expression of pro-oxidative and oxidative stress hallmark genes and (3) the GSH metabolism were investigated and compared between all genotypes.

Out of the results of these objectives, we aim to build a model integrating the link between the oxidative challenge and ethylene biosynthesis and signalling during sublethal Cd exposure in *A. thaliana* leaves.

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Chapter 3

Cadmium-induced ethylene production and responses in *Arabidopsis thaliana* rely on *ACS2* and *ACS6* gene expression

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Abstract

Background

Anthropogenic activities cause metal pollution worldwide. Plants can absorb and accumulate these metals through their root system, inducing stress as a result of excess metal concentrations inside the plant. Ethylene is a regulator of multiple plant processes, and is affected by many biotic and abiotic stresses. Increased ethylene levels have been observed after exposure to excess metals but it remains unclear how the increased ethylene levels are achieved at the molecular level. In this study, the effects of cadmium (Cd) exposure on the production of ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and on the expression of the ACC Synthase (*ACS*) and ACC Oxidase (*ACO*) multigene families were investigated in *Arabidopsis thaliana*.

Results

Increased ethylene release after Cd exposure was directly measurable in a system using rockwool-cultivated plants; enhanced levels of the ethylene precursor ACC together with higher mRNA levels of ethylene responsive genes: *ACO2*, *ETR2* and *ERF1* also indicated increased ethylene production in hydroponic culture. Regarding underlying mechanisms, it was found that the transcript levels of *ACO2* and *ACO4*, the most abundantly expressed members of the *ACO* multigene family, were increased upon Cd exposure. ACC synthesis is the rate-limiting step in ethylene biosynthesis, and transcript levels of both *ACS2* and *ACS6* showed the highest increase and became the most abundant isoforms after Cd exposure, suggesting their importance in the Cd-induced increase of ethylene production.

Conclusions

Cadmium induced the biosynthesis of ACC and ethylene in *Arabidopsis thaliana* plants mainly via the increased expression of *ACS2* and *ACS6*. This was confirmed in the *acs2-1acs6-1* double knockout mutants, which showed a decreased ethylene production, positively affecting leaf biomass and resulting in a delayed induction of ethylene responsive gene expressions without significant differences in Cd contents between wild-type and mutant plants.

Keywords

1-aminocyclopropane-1-carboxylic acid, *acs2-1acs6-1* knockout-mutant, *Arabidopsis thaliana*, cadmium, ethylene, gene expression

3.1 Introduction

Industrial activities and the application of fertilisers, pesticides and sewage sludge in agriculture have contributed to the dispersion of toxic metals, such as cadmium (Cd), in all ecosystem compartments worldwide (Järup and Akesson, 2009). Growing on contaminated soils, plants can take up and accumulate Cd through their root system and transport it to the aboveground plant parts (Clemens et al., 2002; DalCorso et al., 2008). Cadmium bioaccumulation ultimately leads to the introduction of Cd into the food chain, eliciting threats to the public health, even when present at trace concentrations (Clemens et al., 2013; Cuypers et al., 2010; Gallego et al., 2012). Consequently, the reorientation from agricultural to non-food crops is occurring in contaminated areas. These crops are selected for their metal resistance and accumulation capacity, with the final objective to stabilise and clean the soils in a process called phytoremediation (Ruttens et al., 2011; Vangronsveld et al., 2009; Weyens et al., 2012; Witters et al., 2009).

Cadmium is a highly phytotoxic, non-essential element that reduces plant growth and inhibits photosynthesis. Cadmium-induced phytotoxicity is a result of cellular and molecular interactions such as: (1) inactivating and/or denaturing biomolecules by binding their functional groups, (2) replacing essential elements (co-factors) showing chemical similarities and (3) increasing the production of reactive oxygen species (ROS), hereby affecting the cellular redox state (Cuypers et al., 2012; Gallego et al., 2012; Gratão et al., 2005; Hall, 2002; Hirt, 2009).

Phytohormones are known to be affected by multifarious biotic and abiotic stress conditions (e.g. toxic metals) and play important roles as signal molecules, integrating developmental programs and responses to environmental stimuli (Arteca and Arteca, 2007; Cao et al., 2009; Maksymiec, 2007). The gaseous hormone ethylene is involved in multiple molecular, biochemical and physiological processes during the entire lifecycle of the plant and has also been related to enhanced ROS accumulation (Bouchez et al., 2007; Montero-Palmero et al., 2014). A relatively simple metabolic pathway controls the biosynthesis of ethylene (Argueso et al., 2007). Methionine, the biological precursor of ethylene, is converted to S-adenosylmethionine (SAM) by SAM Synthetase. 1-aminocyclopropane-1-carboxylic acid (ACC) Synthase (ACS) uses SAM as a

substrate to form ACC. This is mostly the rate-limiting step in the biosynthesis of ethylene. ACC is oxidised to ethylene by ACC Oxidase (ACO), with CO_2 and cyanide as by-products (Argueso et al., 2007; Lin et al., 2009; Vandenbussche et al., 2012). In *Arabidopsis thaliana*, both ACS and ACO are encoded by multigene families, regulated at the transcriptional level by developmental as well as environmental signals (García et al., 2010; Ramonell et al., 2002; Tsuchisaka and Theologis, 2004; Yamagami et al., 2003). In addition, ACS proteins can also be post-translationally modified (e.g. phosphorylation), influencing their stability (Yoshida et al., 2005).

Ethylene is often considered as the 'stress hormone', modulating multiple defence responses to stresses such as wounding, hypoxia, drought and excess ozone or salt but for example also partially controlling mycorrhizal development and colonisation (Cao et al., 2009; Fracetto et al., 2013; Lin et al., 2009; Voesenek and Sasidharan, 2013; Wang et al., 2002; Zsögön et al., 2008). It is known that an increasing ethylene production ensued by regular signal transduction can inhibit plant development and accelerate senescence and abscission processes (Argueso et al., 2007; Monteiro et al., 2011; Vandenbussche et al., 2012). Hence, a better understanding of the metal-induced effects on the ethylene biosynthesis pathway improves our knowledge on plant metal resistance, which can be implemented in future research concerning the phytoremediation of contaminated soils.

Although the responses of ethylene production of plants to different toxic metals have already been investigated many times, the mechanistic basis remains unclear (Abeles et al., 1992; Arteca and Arteca, 2007; Cao et al., 2009; Gratão et al., 2009; Gratão et al., 2012). It is indeed well known that the effect of exposure to metals on ethylene production is metal and concentration specific (Abeles et al., 1992). Mertens et al. (1999) observed an increasing ethylene production in 7-day-old *A. thaliana* plants exposed to 25 – 500 µM copper (Cu) and zinc (Zn) for up to 6 hours. Lequeux et al. (2010), on the other hand, did not observe an effect on ethylene production in 9-day-old *A. thaliana* plants exposed to 50 µM Cu for 24 hours. In addition, Groppa et al. (2003) reported that metal-induced effects on ethylene production are also species-specific. A 14 hours exposure to 1 mM of either Cd or Cu increased the ethylene production in 4-week-old wheat leaves, whereas in sunflower leaves only Cu enhanced the

ethylene production. Rodríguez-Serrano et al. (2009) detected a higher ethylene production in 14-day-old pea plants exposed to 50 µM Cd for 14 days. Exposure to 400 µM Cd or Cu, but not Zn nor nickel (Ni), differently induced ethylene production in various plant parts of *A. thaliana* (Arteca and Arteca, 2007). The effect of these different metals on the ethylene release was also inversely proportional to the age of the plant parts.

Whereas previous studies only investigated the effect of metals on the ethylene production levels, the aim of the present study is to unravel the mechanisms of Cd-enhanced ethylene biosynthesis. Therefore, we characterised the molecular basis of this response in *A. thaliana* plants exposed to environmentally realistic Cd concentrations. We hypothesised that Cd induces ethylene biosynthesis through alterations in the expression of genes encoding the ACS enzymes, the rate-limiting step in ethylene biosynthesis, yielding the basis of the Cd-induced ethylene production that may influence acclimation to Cd stress.

3.2 Methods

3.2.1 Plant material, culture, treatment and sampling

Arabidopsis thaliana (Columbia ecotype) wild-type and *acs2-1acs6-1* double KO-mutant seeds (N16581) were obtained from the European Arabidopsis Stock Centre (NASC). These mutant plants were described by Tsuchisaka et al. (2009) and they were checked for homozygosity by PCR as instructed.

After surface sterilisation, seedlings were cultivated using a modified Hoagland nutrient solution either (1) on hydroponics according to Smeets et al. (2008), but using purified sand or (2) on rockwool plugs. Established growth conditions for both culturing systems were 12 h photoperiod with day/night temperatures of respectively 22/18 °C and 65 % relative humidity. A combination of blue, red and far-red led modules (Philips Green-Power LED modules) was used to simulate the photosynthetic active radiation (PAR) spectrum of sunlight with a photosynthetic photon flux density of 170 µmol m^{-1} s^{-1} at the leaf level (Keunen et al., 2011).

Three-week-old plants grown on hydroponics were exposed to 5 or 10 µM $CdSO_4$ at the root level (except for control plants). These sublethal concentrations are commonly found in the pore water of moderately contaminated soils and were also applied in previous hydroponic growth experiments (Krznaric et al., 2009). After 24 or 72 h of exposure, whole root and shoot systems were separated, sampled and snap frozen in liquid nitrogen prior to storage at -70 °C and further analyses except for quantification of Cd contents. Biological replicates for each measured parameter (number of replicates displayed in table and figure legends) were sampled from various pots of the same conditions to avoid within pot correlation (Smeets et al., 2008).

For ethylene emission analysis using the rockwool (Grodan Delta, Grodan, Roermond, The Netherlands) cultivation system, seven plants were grown per plug (5 cm diameter, 3.5 cm height), pre-moistened with the same modified Hoagland nutrient solution as in hydroponics. The plugs were positioned in modified Aratrays (Arasystem, Beta Tech, Ghent, Belgium) and placed in lightproof containers filled with 1 L modified Hoagland nutrient solution, leaving only the surface of the plugs, and later the shoots of the plants visible (Supplemental file 3.1). The nutrient solution was refreshed twice a week.

3.2.2 Quantification of Cd contents

Roots and leaves of hydroponically grown plants were harvested. Roots were washed for 15 min with ice-cold 10 mM $Pb(NO_3)_2$ and rinsed in distilled water at 4 °C to exchange surface-bound elements (Cuypers et al., 2002). Leaves were rinsed with distilled water. Samples were oven-dried at 80 °C and digested in HNO_3 (70-71 %) in a heat block. Cadmium concentrations in the extracts were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Perkin-Elmer, 1100B, USA). As references, blanks (HNO_3 only) and certified standard samples (NIST Spinach (1570a)) were analysed. For rockwool-cultivated plants, leaves were processed identically. In this system, roots were not freely available and could therefore not be analysed.

3.2.3 Determination of ACC content

Root and leaf samples of hydroponically grown plants were ground under frozen conditions in a Retsch Mixer Mill 2000 (Retsch, Haan, Germany) using stainless steel beads. D_4-ACC (250 pmol, Olchemim Ltd. Olomouc, CZ. Rep.) was added as internal standard for quantification. ACC was extracted by a solid-phase extraction procedure using half the extract (Smets et al., 2003). ACC-conjugates were purified and analysed as ACC after dry acid hydrolysis of the second half of the extract (Chauvaux et al., 1993). Subsequently, both fractions were derivatised with pentafluorobenzyl (PFB) bromide and analysed as PFB-bis-ACC by Negative Ion Chemical Ionisation Gas Chromatography-mass spectrometry (NICI GC-MS) following Smets et al. (2003) (Quattro micro MS/MS, Waters, Manchester, UK, E.E. 70 eV, Emission 200 µA, extraction 10 V, Source 206 µA, GC interface T: 120 °C, CI gas flow 69 mL/min, WCOT CP-Sil 5 C8 Low bleed/MS column, 30 m, 250 µm, film thickness 0.25 µm (Varian), mobile phase helium, T gradient 50 to 250 °C at 25 °C/min) (Netting and Milborrow, 1988). The diagnostic transitions used for Multiple Reaction Monitoring (MRM) were for ACC: 280>112 and 280>167 and for D4-ACC: 284>116 and 284>167 corresponding to their pentafluorobenzyl (PFB-bis-ACC) derivatives. The transitions 280>114 and 284>116 were used for calculating concentrations. Data are expressed in picomoles per milligram fresh weight (pmol mg^{-1} FW^{-1}).

3.2.4 Gene expression analysis

From root and leaf tissues of hydroponically grown plants, disrupted the same way as for the ACC content, RNA was extracted using the RNAqueous® Phenol-free total RNA Isolation Kit (Ambion, Life Technologies, Paisley, UK), according to the manufacturers instructions. RNA concentration and purity was evaluated spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, Wilmington, USA). DNase treatment with the TURBO DNA-free™ Kit (Ambion, Life Technologies, Paisley, UK) was performed to eliminate possible genomic DNA contamination. One µg of the treated RNA per sample was converted to single stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Ambion, Life Technologies, Paisley, UK) according to the manufacturers instructions. A 10-fold dilution of the produced cDNA was prepared in 1/10 diluted TE buffer (1 mM Tris–HCl, 0.1 mM Na2-EDTA, pH 8.0; Sigma–Aldrich, Belgium) and stored at -20 °C. Quantitative real-time PCR was performed in an optical 96-well plate with the 7900HT Fast Real-Time PCR System (Life Technologies, Paisley, UK) using SYBR Green chemistry. Gene-specific forward and reverse primers were designed and optimised via the Primer Express software (v2.0, Life Technologies, Paisley, UK). Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C) followed by the generation of a dissociation curve to verify amplification specificity. Reactions contained 2 µL diluted cDNA template (or RNase-free H_2O for the 'no template controls'), 5 µL 2x Fast SYBR® Green Master Mix (Life Technologies, Paisley, UK), forward and reverse primers (300 nM each, unless otherwise mentioned in Supplemental file 3.2) and 2.4 µL RNase-free H_2O in a total volume of 10 µL. The specificity of the used primer pairs was checked *in silico* using Blast (http://www.arabidopsis.org/Blast/index.jsp) and after qPCR by verifying single peaks on the dissociation curve. In addition, primer efficiency (e) was evaluated on a standard curve generated using a twofold dilution series of a mixed sample over at least five dilution points and verified to be higher than 80 % (e = 10^(-1/slope)). In supplemental file 3.2, all gene annotations, primer sequences and primer efficiencies are shown. Gene expression levels were calculated according to the $e^{-\Delta Cq}$ method relative to the sample with the highest expression (minimum Cq). The data obtained were normalised using the geometric average of the $2^{-\Delta Cq}$ values of three stable reference genes selected

out of a set of 10 (Remans et al., 2008) by geNorm (v3.5) and Normfinder (v0.953) algorithms (Andersen et al., 2004; Vandesompele et al., 2002). According to the experimental set-up the most stable reference genes were used to determine sample-specific normalisation factors (Supplemental file 3.3).

To calculate the relative abundance of distinct gene family members, the expression level of each family member was determined for the control sample panel (0 h, 0 µM Cd) relative to the highest expressed family member. This yields a relative abundance factor for each member of the gene family, which is used in the calculation of its relative abundance in the kinetic Cd exposure experimental setup. Subsequently, the changes in expression level for each member of a gene family were determined in function of the exposure time and Cd concentration applied and set relatively to the control (0 h, 0 µM Cd).

3.2.5 Determination of ethylene production

Rockwool plugs containing three-week-old plants or blank plugs as mock controls were individually transferred into closed glass cuvettes (7 cm in diameter, 7 cm high) kept at 12/12 light/dark regime and exposed at dawn to 0, 10, 25 or 100 µM $CdSO_4$ by injection in the middle of the rockwool plug. The cuvettes were flushed with hydrocarbon free air (Air Liquide, Aalter, Belgium) every 24 h. The ethylene in the headspace was detected by an ETD-300 Photo-acoustic ethylene detection system (Sensor Sense, Nijmegen, The Netherlands) and analysed using microcal Origin software (Northampton, Massachusetts). Ethylene standard mixtures for calibration were supplied by AirLiquide. Ethylene production was calculated in picolitres per milligram fresh weight per hour (pL mg^{-1} FW^{-1} h^{-1}).

3.2.6 Statistical analysis

The datasets were analysed via the linear model procedure in R (R Development Core Team., 2012). Both normality (Shapiro-Wilk test) and homoscedasticity (residue plot) were checked; transformations were applied when necessary to approximate normality. Normally distributed data were analysed using the one- or two-way ANOVA procedure. Tukey–Kramer adjustment for multiple comparisons was applied to obtain corrected p-values. The statistical analyses of

non-normally distributed data were based on the non-parametric Kruskal–Wallis test followed by the post hoc pairwise Wilcoxon rank sum test.

3.3 Results

3.3.1 Biosynthesis of the ethylene precursor ACC in wild-type plants

The immediate precursor of ethylene, ACC, can be reversibly conjugated to malonyl-ACC or γ-L-glutamyl-ACC (MACC, GACC) (McDonnell et al., 2009; Plett et al., 2009). In most cases, the presence of ACC reflects the activity of the rate-limiting step in ethylene biosynthesis that eventually determines the hormonal content. In order to evaluate the effect of Cd on ethylene biosynthesis, we first estimated the concentration of free as well as conjugated ACC in wild-type *A. thaliana* plants exposed to Cd.

In roots, exposure to 5 μM Cd had no significant effect on the concentration of either the free or the conjugated ACC (Fig. 3.1 A). Exposure to 10 μM Cd on the other hand increased the concentration of both forms of ACC (Fig. 3.1 A). While the concentration of free ACC was comparable after 24 h and 72 h of exposure to Cd, the conjugated ACC content continued to increase towards the later time point. The relative impact of Cd on the content of free ACC was higher compared to conjugated ACC (Fig. 3.1 A).

In leaves, both concentrations of Cd induced the same significant increase in free ACC, with a maximum content after 24 h of exposure (Fig. 3.1 B). Although the abundance of conjugated ACC in general was always higher, the free ACC content was significantly more affected by Cd, whereas conjugated ACC content in the leaves only showed an increasing trend after 24 h of exposure (Fig. 3.1 B).

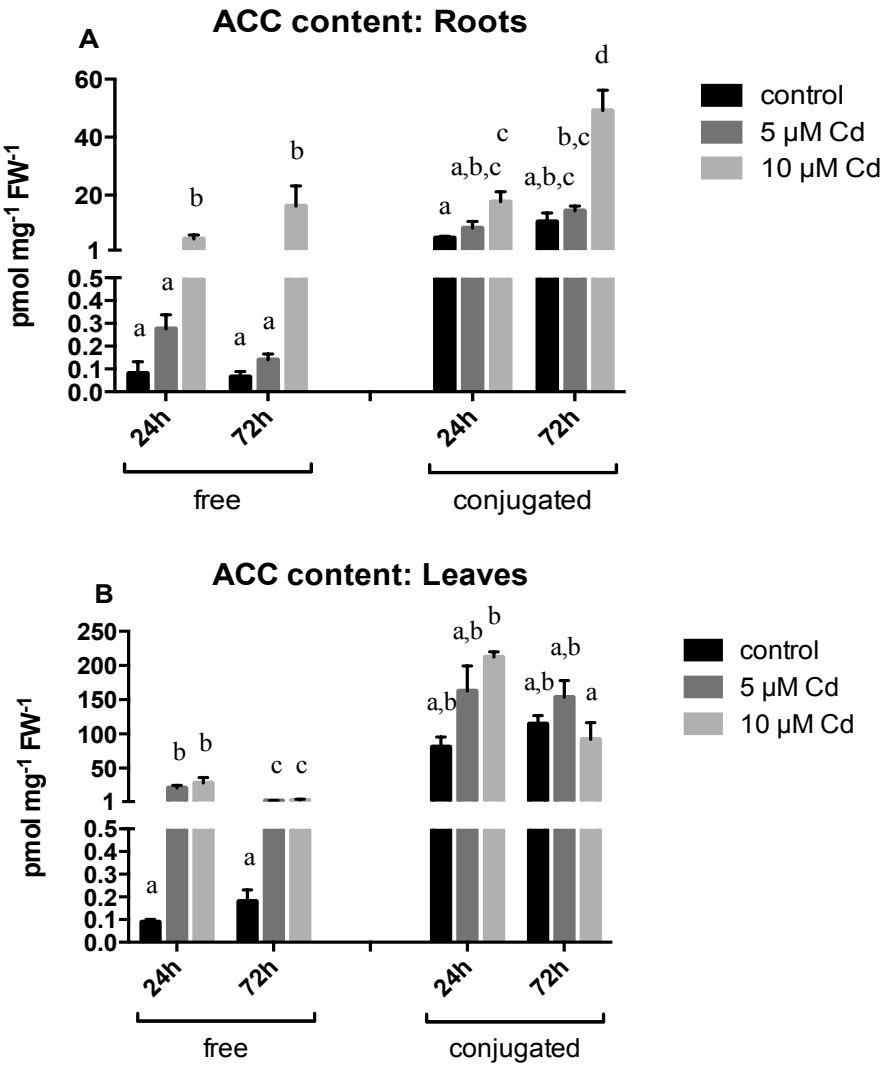

Figure 3.1 ACC content. *ACC content (free and conjugated; pmol mg^{-1} FW^{-1}) in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data are given as mean ± s.e. of at least 5 biological replicates. The letters a-d (A) & a-c (B) represent groups with significantly different amounts of ACC (Tukey's test: $p < 0.05$; except for free ACC content in the roots, Wilcoxon rank sum test: $p < 0.05$). Statistics was performed separately for free and conjugated ACC.*

3.3.2 Expression of genes involved in ACC and ethylene biosynthesis

ACC is produced by ACS enzymes, originating from a multigene family. Within this 12-membered family, *ACS3* is a pseudogene and *ACS10* and A*CS12* encode aminotransferases with different functions (Yamagami et al., 2003). This leaves 9 actual *ACS* genes, whose induced expression may contribute to increased ACC synthesis, that were analysed in this study. The expression of *ACS9* was generally below detection limit in our experimental conditions, confirming earlier observations that *ACS9* transcription is nearly absent in vegetative tissues (Tsuchisaka and Theologis, 2004). Transcript levels of *ACS1*, only functional as a heterodimer, were also very low under control conditions. Analysis of gene family expression included quantification of the total transcript abundance of all isoforms together, as well as the relative contribution of each member. Supplemental file 3.4 A & B shows the relative expression of the individual gene family members to the untreated controls.

In roots, total *ACS* transcript abundance increased after exposure to Cd in a time- and dose-dependent manner, peaking after 72 h of treatment with 10 µM Cd (Fig. 3.2 A). Induction of the transcript levels of *ACS2*, *ACS6* and *ACS7* seemed to particularly contribute to this increased expression level of *ACS* (Fig. 3.2 A). Furthermore, the gene expression of *ACS8* also increased significantly after exposure to 10 µM Cd (Supplemental file 3.4 A), although the relative transcript abundance remained low. In leaves, the highest increase in *ACS* gene expression occurred after 24 h of treatment with Cd (Fig. 3.2 B). The transcript abundance of *ACS2* and *ACS6* was mostly affected upon Cd exposure (Fig. 3.2 B). Expression of *ACS7* and *ACS8* was also slightly, although significantly upregulated (Fig. 3.2 B; Supplemental file 3.4 B). ACS6 was the isoform with the most abundant transcript levels under control conditions in roots and leaves of *A. thaliana*, and was also Cd responsive in both organs (Fig. 3.2 B).

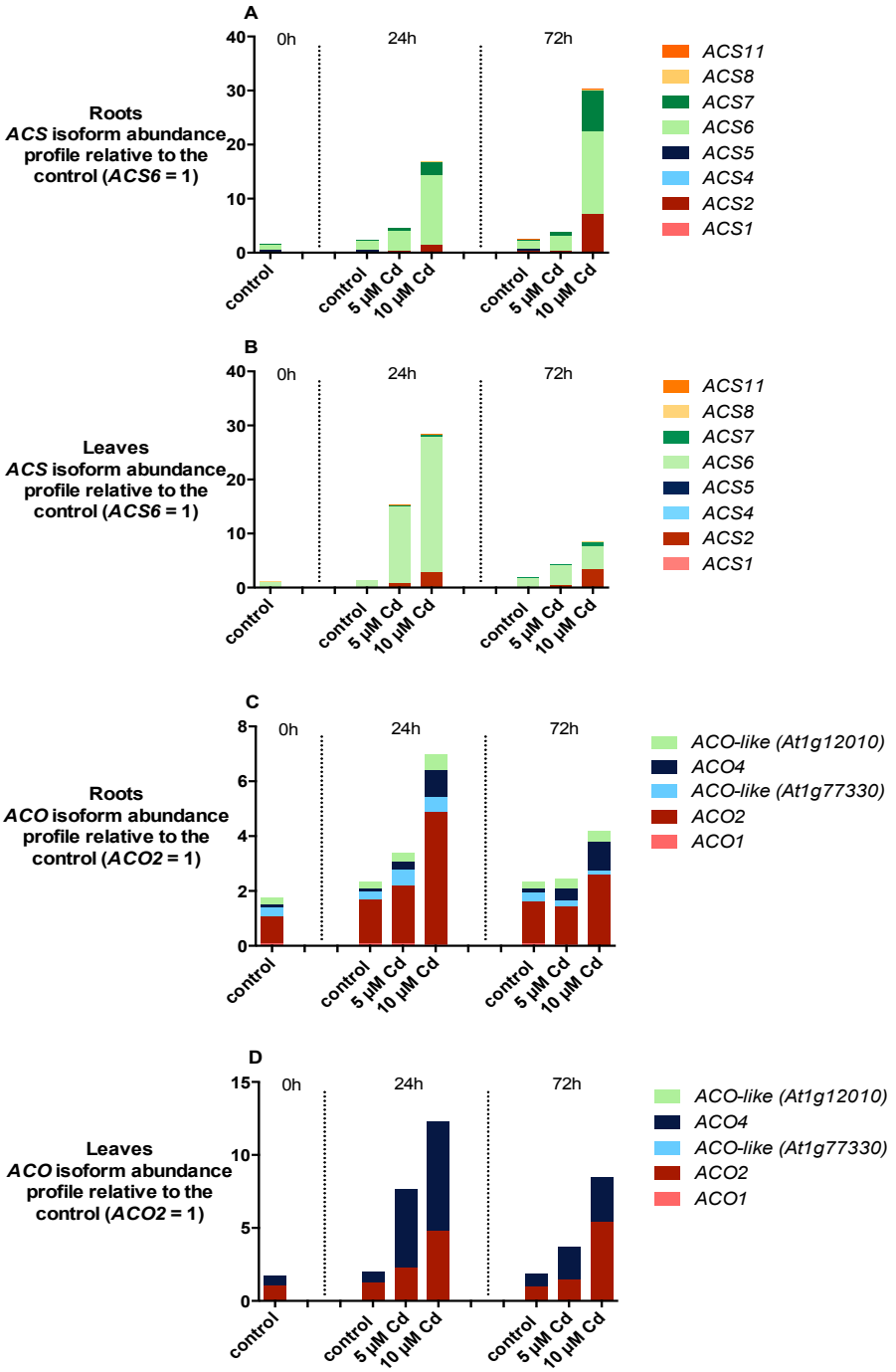
A
Roots
ACS isoform abundance profile relative to the control (ACS6 = 1)
0h
24h
72h
control
control
5 µM Cd
10 µM Cd
control
5 µM Cd
10 µM Cd
ACS11
ACS8
ACS7
ACS6
ACS5
ACS4
ACS2
ACS1
B
Leaves
ACS isoform abundance profile relative to the control (ACS6 = 1)
C
Roots
ACO isoform abundance profile relative to the control (ACO2 = 1)
ACO-like (At1g12010)
ACO4
ACO-like (At1g77330)
ACO2
ACO1
D
Leaves
ACO isoform abundance profile relative to the control (ACO2 = 1)

Figure 3.2 Relative abundance of ACS and ACO multigene family. *Relative abundance of ACS (A-B) and ACO (C-D) multigene family members in roots and leaves of 3-week-old Arabidopsis thaliana plants exposed for 0, 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data represent mean abundance of at least 4 biological replicates relative to the control (0 h, 0 µM $CdSO_4$) and with the abundance of the most highly expressed family member set at 1 under the control condition. (A) Relative abundance of ACS multigene family members in roots. (B) Relative abundance of ACS multigene family members in leaves. (C) Relative abundance of ACO multigene family members in roots. (D) Relative abundance of ACO multigene family members in leaves.*

In addition, gene expression of the 5-membered *ACO* multigene family, which encodes the proteins catalysing the final step of the ethylene biosynthesis, was also analysed (Lin et al., 2009; Tsuchisaka and Theologis, 2004; Yamagami et al., 2003).

The rise in total transcript levels of the *ACO* multigene family reached a maximum after 24 h of exposure to Cd. In roots this was mainly due to the Cd-induced *ACO2* expression, however *ACO4* transcript levels also increased after exposure to 10 µM Cd (Fig. 3.2 C; Supplemental file 3.4 A). In leaves, gene expression of both *ACO2* and *ACO4* increased after treatment with 5 or 10 µM Cd (Fig. 3.2 D; Supplemental file 3.4 B). Hence, these were generally the ACO isoforms with the most abundant transcript levels in both organs after Cd exposure.

3.3.3 Ethylene emission: a comparison between wild-type and *acs2-1acs6-1* mutant plants

The production of ACC by ACS is the rate-limiting step in the ethylene production of *A. thaliana*. Our qRT-PCR data suggests that mainly *ACS2* and *ACS6* contributed to the increased expression of *ACS* genes after exposure to Cd. To verify the importance of these genes for Cd-induced ethylene production, wild-type and *acs2-1acs6-1* double knock-out mutant *A. thaliana* plants were investigated. First, Cd accumulation was compared between wild-type and mutant *acs2-1acs6-1* plants to assess whether genotypic differences in Cd uptake may be present. In hydroponically cultivated plants, the Cd content in roots and leaves of both genotypes increased in a time- as well as dose-

dependent manner (Table 3.1 A). The Cd content in plants treated with 5 µM Cd was similar in roots and leaves. After exposure to 10 µM Cd, roots accumulated twice as much Cd compared to leaves (Table 3.1 A). No significant differences in Cd accumulation were observed between the wild-type and *acs2-1acs6-1* mutant plants.

Table 3.1. Cd content of Arabidopsis thaliana grown in different culture systems.
A comparison of the Cd concentrations (mg kg^{-1} DW^{-1}) in roots and leaves (A) or leaves only (B) of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic (A) or rockwool (B) culture system. Data represent mean ± s.e. of three to six biological replicates. The letters a-b represent groups with a significantly different Cd content after treatment (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time and within each organ. nd: levels below detection limit.

A

Cd content	**Hydroponics**			
	24 h		72 h	
Roots	wildtype	*acs2-1acs6-1*	wildtype	*acs2-1acs6-1*
0 µM $CdSO_4$	nd	nd	nd	nd
5 µM $CdSO_4$	923 ± 16 a	692 ± 54 a	1712 ± 151 a	1327 ± 167 a
10 µM $CdSO_4$	3833 ± 449 b	3079 ± 195 b	6465 ± 476 b	5674 ± 633 b
Leaves	wild-type	*acs2-1acs6-1*	wild-type	*acs2-1acs6-1*
0 µM $CdSO_4$	nd	nd	nd	nd
5 µM $CdSO_4$	976 ± 137 a	883 ± 16 a	1527 ± 106 a	1451 ± 32 a
10 µM $CdSO_4$	1683 ± 100 b	1829 ± 163 b	2989 ± 335 b	3069 ± 74 b

B

Cd content	**Rockwool**			
	24 h		72 h	
Leaves	wildtype	*acs2-1acs6-1*	wildtype	*acs2-1acs6-1*
0 µM $CdSO_4$	nd	nd	nd	nd
5 µM $CdSO_4$	133 ± 7 a	134 ± 8 a	168 ± 18 a	149 ± 41 a
10 µM $CdSO_4$	222 ± 16 b	128 ± 37 a	192 ± 30 a	288 ± 48 a

The ethylene emission of whole plants was measured as described by Woltering et al. (Woltering et al., 1988), using a rockwool cultivation system. Since Cd uptake in rockwool cultivated plants may differ from that in hydroponics, which in turn may affect ethylene production, both growth systems were also compared for Cd accumulation. Therefore, the internal Cd concentration in the leaves of these plants was compared with the previous results of the hydroponically grown plants (Table 3.1). Overall, the Cd uptake in rockwool-cultivated plants was six- to fifteen times lower compared to hydroponically grown plants. Cadmium accumulation in mutant plants exposed to 5 µM Cd did not significantly differ from the wild type. On the other hand, 24 h of exposure to 10 µM Cd led to a significantly lower Cd content in the mutant plants, but no significant differences were observed after 72 h of exposure to 10 µM Cd (Table 3.1 B). In order to reach internal Cd concentrations comparable to those attained in hydroponically grown plants, higher external Cd concentrations were applied in the rockwool cultivation system to provoke Cd-induced ethylene production. Consequently, concentrations of 10 µM, 25 µM or 100 µM Cd were applied. Exposure to the various Cd concentrations always significantly increased the ethylene emission in wild-type plants. In the *acs2-1acs6-1* double KO-mutants on the other hand, at none of the applied concentrations a Cd-induced increase in ethylene emission was observed (Fig. 3.3).

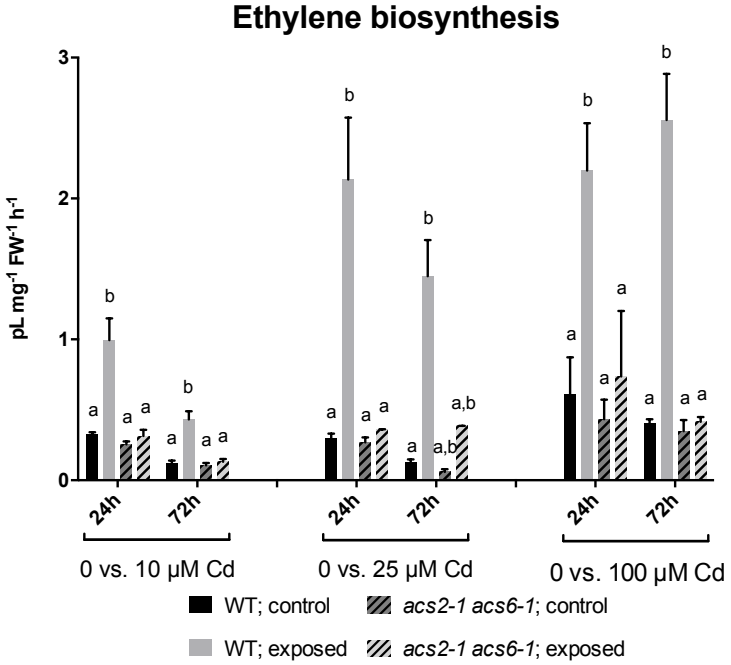

Figure 3.3 Ethylene emission. *A comparison of the ethylene emission (pL mg^{-1} FW^{-1} h^{-1}) in 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to 10, 25 or 100 µM $CdSO_4$ or grown under control conditions in a rockwool culture system. Data are shown as mean ± s.e. of at least 3 biological replicates. The letters a-b represent groups with a significantly different ethylene production (Tukey's test: $p < 0.05$; except 25 µM $CdSO_4$ - 72 h, Wilcoxon rank sum test: $p < 0.05$). Statistics was performed separately for each Cd concentration and within each exposure time.*

As already mentioned, ethylene is a modulator of growth and developmental stages during the entire life cycle of the plant and it is responsible for the induction of cell senescence. Because of the difference in ethylene production between both genotypes, the biomass of roots and leaves was compared after Cd exposure. Furthermore, the growth inhibition caused by exposure to Cd was determined in both organs relative to the controls within each genotype.

Neither the wild-type nor the mutant plants showed a significant decrease in root biomass after 24 h of exposure to 5 or 10 µM Cd (Fig. 3.4 A). Exposure to either of both Cd concentrations during 72 h did induce a significant reduction in root biomass in both genotypes. The growth inhibition of the Cd-exposed roots relative to the control roots was always higher in the wildtype compared to the *acs2-1acs6-1* mutants (Fig. 3.4 A).

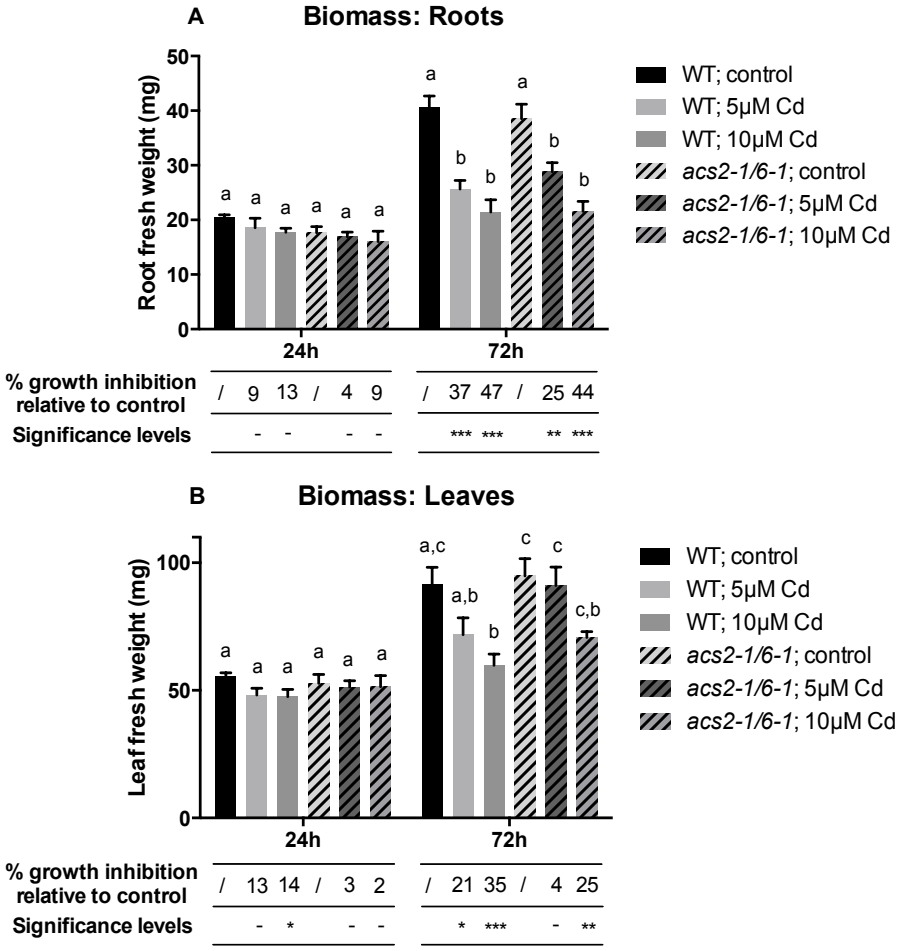

Figure 3.4 Biomass & growth inhibition. *A comparison of the fresh weight biomass and growth inhibition (mg) of roots (A) and leaves (B) of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Biomass: Data shows mean ± s.e. of at least 4 biological replicates. The letters a-c represent groups with a significantly different biomass (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time. Growth inhibition: Data shows mean ± s.e. of at least 4 biological replicates relative to the control within each exposure time and genotype. Significance levels: − = no significant difference; * = $p < 0.1$; ** = $p < 0.05$; *** = $p < 0.01$ (Tukey's test). Statistics was performed separately within each exposure time and genotype.*

In leaves, no significant differences in biomass were observed after 24 h of exposure to 5 or 10 µM Cd between both genotypes. Nevertheless, the growth was significantly inhibited in wild-type plants after 24 h of exposure to 10 µM Cd, which could not be observed in the mutant plants (Fig. 3.4 B). Exposure during 72 h to either of both concentrations of Cd did not induce a significant leaf biomass reduction in the *acs2-1acs6-1* mutant plants. On the contrary, there was a significant decrease in leaf biomass of wild-type plants exposed to 10 µM Cd (Fig. 3.4 B). Moreover, a significant difference in biomass between the wild-type and mutant plants exposed to 5 µM Cd was observed, which was confirmed by the growth inhibition data. Similar to the roots, the growth inhibition of the leaves in Cd-exposed plants was always higher in the wildtype compared to the *acs2-1acs6-1* mutants (Fig. 3.4 B).

3.3.4 Ethylene responsive genes: a comparison between wild-type and *acs2-1acs6-1* mutant plants

To investigate whether the differences in ethylene production between *acs2-1acs6-1* mutants and wild-type plants were sufficient to provoke a differential ethylene response, expression of primary ethylene responsive genes was measured in both genotypes. The genes encoding for the ethylene receptor *ETR2*, the biosynthesis enzyme *ACO2* and the ethylene response factor *ERF1* are known to be ethylene responsive (Vandenbussche et al., 2012).

In roots, Cd exerted the greatest effect on the expression of all three genes after 24 h of exposure. The expression of *ACO2* was significantly higher in wild-type plants as compared to the mutants after 24 h exposure to both concentrations. However, after 72 h of Cd exposure there were no significant differences between wild-type and mutant plants (Fig. 3.5 A). For *ETR2* expression, a similar pattern as for *ACO2* was observed, except after 24 h exposure to 10 µM Cd, no significant differences between both genotypes were observed (Fig. 3.5 C). The expression of *ERF1* in the wildtype was significantly higher compared to the mutant after exposure to 5 µM Cd, and after 72 h of exposure to 10 µM Cd (Fig. 3.5 E).

In leaves, the expression of these three genes was always significantly higher in wild-type plants after 24 h exposure to both Cd concentrations compared to the mutants (Fig. 3.5 B, D, F). After 72 h of exposure there were less significant

differences, only *ACO2* showed significantly higher transcript levels in wild-type plants compared to the mutants exposed to 5 µM Cd (Fig. 3.5 B, D, F).

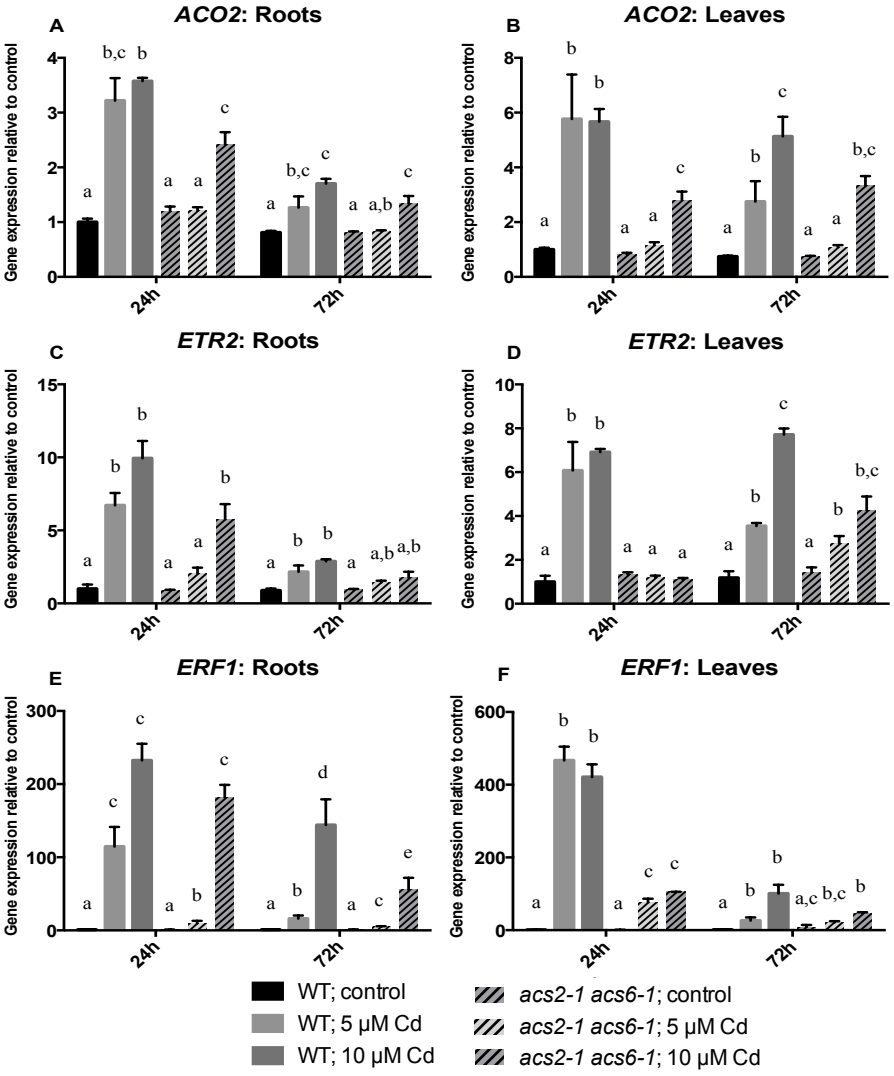

Figure 3.5 Relative expression of ethylene responsive genes. *A comparison of the relative expression of* ACO2 *(A-B),* ETR2 *(C-D) and* ERF1 *(E-F) in roots and leaves of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data shows mean ± s.e. of at least 4 biological replicates relative to the control (24 h, 0 µM $CdSO_4$). The letters a-d represent groups with a significantly different gene expression (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time.*

3.4 Discussion

3.4.1 Cadmium stress increases ethylene production in *Arabidopsis thaliana*

Ethylene is a well-known regulator of miscellaneous plant responses, and is affected by many biotic and abiotic stresses (Cao et al., 2009; Dugardeyn and Van Der Straeten, 2008; Lin et al., 2009). Also after exposure to excess metals, increased ethylene levels have been observed (Abeles et al., 1992; Arteca and Arteca, 2007; Groppa et al., 2003; Mertens et al., 1999; Rodríguez-Serrano et al., 2009). Ethylene is enzymatically synthesised from SAM in two steps, with ACS, encoded by a multigene family, as the rate-limiting enzyme (Lin et al., 2009; Vandenbussche et al., 2012). Still, it remains unclear how an increase in ethylene release after toxic metal exposure is achieved at the molecular level. Therefore, in the present study, a kinetic approach was adopted to investigate the effects of Cd exposure on ACC and ethylene production in *A. thaliana* as well as the influence of Cd on the expression of the *ACS* and *ACO* multigene families involved in ethylene biosynthesis.

The immediate precursor of ethylene, ACC, exists in a free (active) as well as conjugated (inactive) form. Although being reversible to a certain extent, the conjugation of ACC makes it, at least temporarily, unavailable for the ethylene biosynthesis pathway (Plett et al., 2009). The accumulation of conjugated ACC could serve to optimise free ACC levels as a substrate for ACO, converting it to ethylene. Deconjugation can subsequently restore free ACC levels to avoid depletion. In contrast with previous studies, we quantified both forms of ACC separately, not only focussing on free ACC. Exposure to 5 or 10 µM Cd induced the accumulation of free as well as conjugated ACC in roots and leaves of wild-type *A. thaliana* plants grown in hydroponics (Fig. 3.1). This can explain the observed increase in ethylene release under Cd stress (Fig. 3.3). In roots, the overall ACC content is lower compared to leaves. This could be due to a lower production rate or transportation of ACC from the roots to the leaves (Shiu et al., 1998). The fact that exposure to 5 µM Cd did not significantly increase the ACC content in roots could be explained by the rate-limiting character of this step. Most of the ACC could immediately be converted into ethylene, as observed from the ethylene biosynthesis data (Fig. 3.3). This hypothesis can also be confirmed by the increase in expression of ethylene responsive genes in

roots after exposure to 5 µM Cd (Fig. 3.5 A, C, E). Previous studies also reported increasing ACC contents in roots and leaves of tomato plants after three weeks of growth on salinised medium (Albacete et al., 2008; Dodd and Pérez-Alfocea, 2012; Ghanem et al., 2008). Likewise, Siddikee et al. (2011) observed higher ACC levels in roots of two-week-old red pepper plants exposed to salt stress for one week. On the contrary, Ben Salah et al. (2013) reported a decrease in ACC content after 3 weeks of salt stress in roots and leaves of the salt-tolerant *Medicago ciliaris*. In contrast with our findings, Han et al. (2013) did not find a clear correlation between Cd exposure and ACC content in leaves of the halophyte *Kosteletzkya virginica*. Three weeks of exposure to 5 µM Cd did not increase the ACC concentration, addition of 50 mM NaCl together with Cd even decreased the ACC content. This points to different responses in salt tolerant and sensitive species.

Genes known to be responsive to elevated ethylene levels showed an increase in expression in hydroponically cultivated plants exposed to 5 or 10 µM Cd (Fig. 3.5), also indicating an augmentation of ethylene biosynthesis. The latter was verified in our study in wild-type plants grown on rockwool, displaying a dose-dependent increase in ethylene production after 24 and 72 h of exposure to 10, 25 and 100 µM Cd (Fig. 3.3). Consequently both the hydroponic and rockwool growth system clearly support a Cd-induced ethylene biosynthesis.

3.4.2 The stress related ACS2 and ACS6 are the main isoforms involved in Cd-induced ethylene production

To further unravel these findings, the expression of genes encoding the enzymes involved in ethylene biosynthesis, ACS and ACO were analysed. Hitherto, few studies investigated the effect of toxic metals on the differential expression of the *ACO* multigene family members. Srivastava et al. (2007) reported a lead-induced upregulation of a putative *ACO* gene in *Sesbania drummondii*. Kim et al. (1998) observed increased *ACO1* and *ACO3* transcript levels in *Nicotiana glutinosa* after 48 h of exposure to Cu. Dorling et al. (2011) on the other hand did not detect differences in *ACO* transcript levels of *Trifolium repens* after 9 days of excess manganese (Mn). To the best of our knowledge, this is the first time the effect of toxic sublethal Cd exposure on *ACO* gene expression was investigated in *A. thaliana*. The transcript levels of *ACO2* and *ACO4*, the two

most abundant members of the *ACO* multigene family, coding for the enzymes responsible for the conversion of ACC to ethylene (Fig. 3.2 C & D) increased in a dose-dependent manner. These results corroborate the conclusions of Ruduś et al. (2012), who observed upregulations of various *ACO* genes after exposure to abiotic (wounding, flooding) and biotic (pathological infection) stresses, serves as a good ethylene production indicator.

The rate-limiting step in ethylene biosynthesis, however, is the conversion of SAM to ACC by ACS (Lin et al., 2009). The expression of eight different genes coding for the ACS isoforms was assessed (Fig. 3.2 A & B). The maximum increases in expression of *ACS* genes, after 72 h or 24 h of exposure to Cd for respectively roots and leaves, correlated well with the ACC content in both organs (Fig. 3.1). Cadmium exposure particularly increased the abundance of *ACS2* and *ACS6* transcript levels. These two isoforms are the only active type 1 ACS proteins, making them phosphorylation targets of mitogen-activating protein kinase (MAPK) MPK3/MPK6. This posttranslational modification reduces the turnover by the 26S proteasome degradation machinery, prolonging the half-life of the ACS enzymes (Lin et al., 2009; Skottke et al., 2011; Yoo et al., 2009). In addition, MPK3 and MPK6 are also capable of inducing the transcriptional activity of *ACS2* and *ACS6* via WRKY33 (Li et al., 2012). The involvement of MAPK signalling in plants under metal stress has been reported several times (Opdenakker et al., 2012). Jonak et al. (2004) showed that SAMK/SIMK, the *Arabidopsis* orthologues of MPK3/MPK6 in *Medicago sativa*, were activated after exposure to excess Cd or Cu ions. In *A. thaliana*, MPK3/MPK6 activity and mRNA levels were also induced after exposure to Cd (Jin et al., 2013; Liu et al., 2010; Opdenakker et al., 2012). Various other abiotic stresses are also known to elevate ethylene biosynthesis through induction of different *ACS* transcript levels in *A. thaliana* (Argueso et al., 2007). Interestingly, ACS2 and ACS6 very often appear to regulate the production of stress ethylene in *A. thaliana*. *ACS6* transcript levels were shown to be elevated after exposure to ozone, Li (lithium), Cu, salt stress, … (Arteca and Arteca, 1999; Vahala et al., 1998). *ACS2* gene expression was also upregulated by high salinity (Achard et al., 2006). Peng et al. (2005) reported the induction of *ACS2* and *ACS6* up to 36 h of hypoxic treatments. In addition, the necrotrophic fungus

Botrytis cinerea is known to induce ethylene production through an ACS2 and ACS6 dependent mechanism (Han et al., 2010; Li et al., 2012).

In this study, evidence for the importance of *ACS2* and *ACS6* upregulation in Cd-induced ethylene production was found using the *A. thaliana acs2-1acs6-1* double KO-mutant, which showed a much lower induction of ethylene production. The basal level of ethylene production measured in these mutants may be explained by the presence of other ACS isoforms, which, because of their minor abundance after Cd exposure at transcriptional (except for *ACS7*) or protein level (Supplemental file 3.5), gave rise to low ethylene levels. Many of these other isoforms have been reported to be involved in developmental regulation, rather than stress (Chae, 2003; Thain et al., 2004; Vandenbussche et al., 2003).

No significant differences were found in Cd content between wild-type and mutant plants, indicating that the absence of induction of ethylene production in mutants was not attributable to a decreased Cd uptake (Table 3.1).

With the objective to investigate the consequences for signalling and perception of the lack of ethylene biosynthesis induction, the physiological responses as well as the expression of ethylene responsive genes were measured in *acs2-1acs6-1* mutants and compared to wild-type plants.

As mentioned, no significant differences in root fresh weight were observed between both genotypes (Fig. 3.4 A). In leaves however, Cd induced a significant growth inhibition in the wild-type but not or to a lesser extent in the mutant plants, more specifically at 24 h and 72 h for 10 and 5 µM Cd respectively. This was also reflected in the fresh weight data (Fig. 3.4 B). Hence, within our experimental setup, the negatively affected leaf biomass in wild-type plants was a consequence of Cd-induced ethylene production.

The ethylene biosynthesis gene *ACO2*, the ethylene receptor gene *ETR2* and the ethylene response factor gene *ERF1* are known to have elevated transcript levels in response to ethylene exposure (Hua et al., 1998; Raz and Ecker, 1999; Solano et al., 1998; Zhong et al., 2003). ERF1 is also known to be involved in different stress responses. Cheng et al. (2013) reported that the induction of *ERF1* gene expression after salt and dehydration stress was enhanced by ethylene signalling. Therefore we assumed *ERF1* to be the most indicative ethylene responsive gene of our selection. After exposure of our plants to Cd,

the expression of the three genes was, as mentioned before, significantly higher in roots and leaves of wild-type plants. In the mutants, however, there was evidence for a lower induction of expression of the ethylene responsive genes (Fig. 3.5). The remaining elevated transcript levels of these genes in the roots of mutant plants, especially after exposure to 10 µM Cd, can be explained by the increase in expression of *ACS7*, possibly leading to increased ethylene release (Fig. 3.2 A, Supplemental file 3.5). After 72 h of exposure to Cd the differences in ethylene responsive gene expression between the two genotypes started to fade. Except for the expression of *ACO2* and *ETR2* in the leaves, the transcript levels of the ethylene responsive genes decreased compared to 24 h of exposure to Cd. This could be caused by a transient response of the genes to the ethylene signal, indicating the importance of ethylene in the early response to Cd stress. These results correspond to those of Montero-Palmero et al. (2014), who also observed a transient induction of ethylene responses in mercury (Hg) treated *Medicago sativa* and *A. thaliana* seedlings. The increased *ACO2* and *ETR2* expression in the leaves of both genotypes after 72 h of exposure to Cd could be the result of Cd-induced signalling pathways independent of ACS2 and ACS6.

3.5 Conclusion

In conclusion, Cd induced the biosynthesis of ACC and ethylene in *A. thaliana* plants mainly via the increased expression of *ACS2* and *ACS6*, which was confirmed by the low ethylene levels in *acs2-1acs6-1* double KO-mutants exposed to Cd. Whereas other isoforms still deliver a basal ethylene level, the lack of Cd-induced increase in ethylene production in the double mutants highly diminished the fast-induced expression of ethylene responsive genes, which positively affected the plant leaf biomass.

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SUPPLEMENTAL FILES

Supplemental file 3.1 Rockwool cultivation system.
(A) 7 Arabidopsis thaliana *plants sown on rockwool covered with aluminium foil, positioned in modified Aratrays and placed in lightproof containers filled with 1 L modified Hoagland nutrient solution, leaving only the surface of the plugs visible. (B) Rockwool plugs containing three weeks old plants were transferred to glass cuvettes and connected to the measurement system (the aluminium foil was removed).*

Supplemental file 3.2 Primer and amplicon information.

*Primer and amplicon information (*not measurable by dilution series due to extremely low expression)*

Gene (accession nr)	Forward Primer	Amplicon length (bp)	Intron-spanning	Primer efficiency	Primer concentration
ACS1 (AT3G61510)	F: ACATTTGATTCCGAAATGGCG R: GCTCAGAGCAGTGAAACGACG	91	no	*	x3
ACS2 (AT1G01480)	F: CATGTTCTGCCTTGCGGATC R: ACCTGTCCGCCACCTCAAGT	91	yes	1.99	x1
ACS4 (AT2G22810)	F: GTTACCAAGAACCCTCAAGGCA R: TGTTTTGTGCAAGCCATGACTC	91	yes	2.03	x3
ACS5 (AT5G65800)	F: TTTTGCCTACTCCTTACTATCCTGGA R: TTAGAGCTTGAGCAGTGAATGGG	91	yes	1.89	x3
ACS6 (AT4G11280)	F: TTAGCTAATCCCGGCGATGG R: ACAAGATTCACTCCGGTTCTCCA	92	yes	1.92	x3
ACS7 (AT4G26200)	F: ACGACGCCCTTCTAGTTCCC R: CAGTGGATGGGTACTATTTTCACTCC	91	yes	2.03	x3
ACS8 (AT4G37770)	F: GAAGGCCAATCCATATTTCGG R: CCGACATGAAATCCGCCATA	91	yes	2.02	x3
ACS11 (AT4G08040)	F: AGATGCCTTTCTTATCCCTGCAC R: GCAATGGATAGGAACAATCTCTACTCC	91	yes	2.05	x1
ACO1 (AT2G19590)	F: TTGCTACGTTTTACAATCCGGC R: AGGTAGTCTTGAAAACGGTAGCCA	91	yes	1.93	x1
ACO2 (AT1G62380)	F: TCTACGTTCGTCACCTCCCTCA R: CTCTTACCAAAGTCTTTCATGGCC	91	yes	2.01	x1
ACO-like (AT1G7733)	F: TGTTCAGCCTCTACCTAATGCCA R: CCTGTGCCACGCACTCTTGTA	91	yes	1.97	x1
ACO4 (AT1G05010)	F: CTCCGATGTCCCTGATCTCG R: ATCCAGTAGCTCCTCCGACAACT	91	yes	1.95	x1
ACO-like (AT1G1201)	F: GCATTCCATTGTCATCAACCTTG R: TTTCTGGGTCATCACACGGTG	91	yes	2.06	x1
ETR2 (AT3G23150)	F: TTCGAACCGGGCAGTTACAC R: AATGGCGGTAAGGCAATCG	91	no	1.87	x1
ERF1 (AT3G23240)	F: TCCTCGGCGATTCTCAATTTT R: CAACCGGAGAACAACCATCCT	91	no	1.89	x2

Supplemental file 3.3 Reference gene information.

Reference Genes			
Roots		Primer	Results Section
TIP41-like (AT4G34270)	F:	GTGAAAACTGTTGGAGAGAAGCAA	3.2 & 3.4
	R:	TCAACTGGATACCCTTTCGCA	
PPR gene (AT5G55840)	F:	AAGACAGTGAAGGTGCAACCTTACT	3.2 & 3.4
	R:	AGTTTTTGAGTTGTATTTGTCAGAGAAAG	
ACT2 (AT3G18780)	F:	CTTGCACCAAGCAGCATGAA	3.2
	R:	CCGATCCAGACACTGTACTTCCTT	
SAND family (AT2G28390)	F:	AACTCTATGCAGCATTTGATCCACT	3.4
	R:	TGATTGCATATCTTTATCGCCATC	
Leaves		Primer	Results Section
UBC (AT5G25760)	F:	CTGCGACTCAGGGAATCTTCTAA	3.2 & 3.4
	R:	TTGTGCCATTGAATTGAACCC	
TIP41-like (AT4G34270)	F:	GTGAAAACTGTTGGAGAGAAGCAA	3.2 & 3.4
	R:	TCAACTGGATACCCTTTCGCA	
SAND family (AT2G28390)	F:	AACTCTATGCAGCATTTGATCCACT	3.2 & 3.4
	R:	TGATTGCATATCTTTATCGCCATC	

***Supplemental file 3.4 Relative expression of* ACC oxidase and ACC synthase genes.** *Relative expression of ACC oxidase and ACC synthase genes in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data shows mean ± s.e. of at least 4 biological replicates relative to the control within each time point. The colours represent groups with a significantly different expression (red: decrease; green: increase; Tukey's test: $p < 0.05$). Statistics was performed separately for each gene within each exposure time.*

A

Roots

ACC Oxidase

Gene	$CdSO_4$ (µM)	0 h	24 h	72 h
ACO1	0	1.00 ± 0.06	1.00 ± 0.11	1.00 ± 0.07
	5		0.90 ± 0.10	0.79 ± 0.10
	10		0.55 ± 0.14	0.38 ± 0.05
ACO2	0	1.00 ± 0.10	1.00 ± 0.05	1.00 ± 0.11
	5		1.32 ± 0.14	0.88 ± 0.15
	10		3.00 ± 0.34	1.65 ± 0.39
ACO-like (AT1G77330)	0	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.07
	5		2.08 ± 0.24	0.79 ± 0.08
	10		2.03 ± 0.30	0.48 ± 0.06
ACO4	0	1.00 ± 0.10	1.00 ± 0.10	1.00 ± 0.08
	5		2.75 ± 0.22	2.83 ± 0.35
	10		8.47 ± 1.00	7.04 ± 1.50
ACO-like (AT1G12010)	0	1.00 ± 0.09	1.00 ± 0.12	1.00 ± 0.12
	5		1.17 ± 0.23	1.51 ± 0.04
	10		2.18 ± 0.40	1.70 ± 0.20

ACC Synthase

Gene	$CdSO_4$ (µM)	0 h	24 h	72 h
ACS1	0	1.00 ± 0.08	1.00 ± 0.28	1.00 ± 0.18
	5		0.99 ± 0.25	0.92 ± 0.10
	10		1.33 ± 0.47	30.34 ± 12.11
ACS2	0	1.00 ± 0.09	1.00 ± 0.05	1.00 ± 0.04
	5		1.44 ± 0.56	1.23 ± 0.39
	10		7.93 ± 2.93	35.13 ± 10.73
ACS4	0	1.00 ± 0.15	1.00 ± 0.15	1.00 ± 0.34
	5		1.28 ± 0.39	1.44 ± 0.58
	10		2.01 ± 0.14	1.36 ± 0.33
ACS5	0	1.00 ± 0.08	1.00 ± 0.13	1.00 ± 0.08
	5		0.10 ± 0.05	0.10 ± 0.05
	10		0.02 ± 0.01	0.01 ± 0.00
ACS6	0	1.00 ± 0.13	1.00 ± 0.08	1.00 ± 0.07
	5		2.35 ± 0.37	1.73 ± 0.08
	10		8.17 ± 1.69	9.74 ± 2.00
ACS7	0	1.00 ± 0.11	1.00 ± 0.32	1.00 ± 0.17
	5		1.90 ± 0.27	3.28 ± 0.93
	10		10.07 ± 1.11	35.63 ± 4.28
ACS8	0	1.00 ± 0.14	1.00 ± 0.23	1.00 ± 0.22
	5		2.67 ± 1.28	1.69 ± 0.29
	10		22.83 ± 7.71	35.40 ± 10.74
ACS11	0	1.00 ± 0.12	1.00 ± 0.22	1.00 ± 0.13
	5		3.76 ± 0.64	1.67 ± 0.17
	10		5.27 ± 1.31	1.65 ± 0.69

B

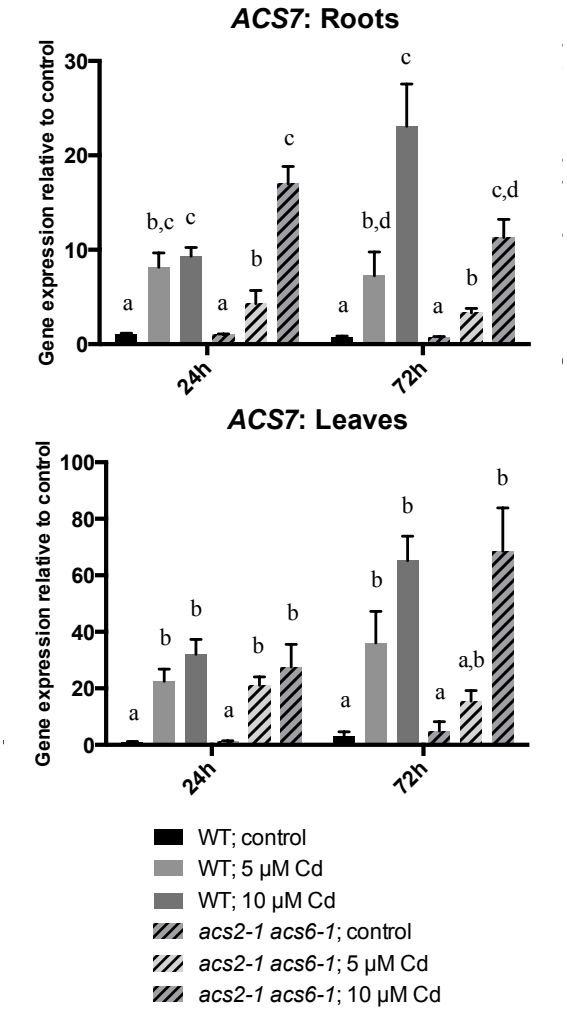
Leaves

ACC Oxidase

Gene	$CdSO_4$ (µM)	0 h	24 h	72 h
ACO1	0	1.00 ± 0.19	1.00 ± 0.22	1.00 ± 0.24
	5		0.42 ± 0.09	0.54 ± 0.15
	10		0.34 ± 0.09	0.46 ± 0.08
ACO2	0	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.08
	5		1.88 ± 0.48	1.53 ± 0.10
	10		3.98 ± 0.71	5.67 ± 0.84
ACO-like (AT1G77330)	0	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.06
	5		0.52 ± 0.12	0.68 ± 0.25
	10		0.51 ± 0.16	0.42 ± 0.13
ACO4	0	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.12
	5		7.17 ± 1.41	2.57 ± 0.33
	10		10.07 ± 1.30	3.56 ± 0.27
ACO-like (AT1G12010)	0	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.01
	5		0.43 ± 0.12	0.60 ± 0.20
	10		0.29 ± 0.05	0.26 ± 0.04

ACC Synthase

Gene	$CdSO_4$ (µM)	0 h	24 h	72 h
ACS1	0	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.21
	5		1.38 ± 0.40	1.02 ± 0.26
	10		2.50 ± 0.57	3.75 ± 0.86
ACS2	0	1.00 ± 0.13	1.00 ± 0.04	1.00 ± 0.39
	5		538.47 ± 156.38	27.26 ± 12.67
	10		2043.65 ± 655.14	254.89 ± 79.48
ACS4	0	1.00 ± 0.19	1.00 ± 0.20	1.00 ± 0.24
	5		0.09 ± 0.00	0.30 ± 0.08
	10		0.04 ± 0.03	0.03 ± 0.01
ACS5	0	1.00 ± 0.16	1.00 ± 0.20	1.00 ± 0.30
	5		0.03 ± 0.01	0.11 ± 0.03
	10		0.07 ± 0.04	0.10 ± -
ACS6	0	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.17
	5		11.76 ± 2.82	2.24 ± 0.63
	10		20.70 ± 2.92	2.60 ± 0.32
ACS7	0	1.00 ± 0.05	1.00 ± 0.11	1.00 ± 0.15
	5		2.26 ± 0.29	1.73 ± 0.65
	10		5.43 ± 1.34	6.81 ± 1.15
ACS8	0	1.00 ± 0.14	1.00 ± 0.26	1.00 ± 0.19
	5		6.93 ± 1.93	2.16 ± 0.64
	10		8.04 ± 3.08	2.50 ± 0.44
ACS11	0	1.00 ± 0.16	1.00 ± 0.12	1.00 ± 0.04
	5		1.50 ± 0.33	1.49 ± 0.57
	10		1.76 ± 0.47	1.09 ± 0.66



Supplemental File 3.5 Relative expression of ACS7. *Relative expression of ACS7 in roots and leaves of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data shows mean ± s.e. of at least 4 biological replicates relative to the control (24 h, 0 µM $CdSO_4$). The letters a-d represent groups with a significantly different gene expression (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time.*

Chapter 4

Ethylene biosynthesis is involved in the early oxidative challenge induced by moderate Cd exposure in *Arabidopsis thaliana*

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Abstract

The stress hormone ethylene is known to be crucial for the survival of adverse environmental stimuli. Cadmium (Cd), a toxic metal, increases ethylene biosynthesis. In this study, wild-type (WT) and *acs2-1acs6-1* double KO-mutant *Arabidopsis thaliana* plants, with an attenuated ethylene response, were exposed to moderate (5 µM) and more severe (10 µM) Cd stress. The short-term influence of the Cd-induced ethylene production on growth and different oxidative stress parameters, and the consequent long-term influence on plant acclimation were investigated. Short-term moderate Cd stress conditions elicited enhanced stress-related responses in WT plants compared to the *acs2-1acs6-1* mutants. The fresh weight of *acs2-1acs6-1* mutant leaves was significantly higher after 72 h exposure to moderate Cd stress. The transcript levels of pro-oxidative and oxidative stress marker genes as well as the expression of *GSH1* and *GSH2*, the enzymes synthesising the antioxidative metabolite glutathione (GSH) were significantly lower in the *acs2-1acs6-1* mutant plants. This resulted in a significantly lower GSH content in the leaves of the *acs2-1acs6-1* mutants. Severe stress apparently overwhelmed the stress signal sensing system of both genotypes, overruling most of these differential responses. Long-term exposure to moderate and severe Cd stress inhibited root and leaf development as well as the reproductive capacity of WT and *acs2-1acs6-1* mutant plants, suggesting ethylene independence. We can conclude that ethylene plays an important role in the early oxidative challenge induced by moderate Cd stress in *A. thaliana*.

Keywords

Arabidopsis thaliana, cadmium, ethylene, oxidative challenge, moderate stress

4.1 Introduction

Plant hormones are crucial signalling molecules integrating multiple developmental programs and responses to environmental stimuli such as biotic and abiotic stresses (Cao et al., 2009; Monteiro et al., 2011; Shan et al., 2012). The gaseous phytohormone ethylene influences various molecular and physiological processes during the lifecycle of the plant (e.g. seed germination, flowering and senescence). It is also considered a 'stress hormone' modulating a diverse array of defence responses (Argueso et al., 2007; Lin et al., 2009; Mittler, 2006).

Exposure to the toxic metal cadmium (Cd) increases ethylene biosynthesis in *Arabidopsis thaliana* through an upregulated expression of 2 stress-sensitive 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) isozymes, ACS2 and ACS6 (Arteca and Arteca, 2007; Schellingen et al., 2014). This enzyme completes the first and rate-limiting step of the ethylene biosynthesis pathway, converting S-adenosylmethionine (SAM) to ethylene's direct precursor, ACC (Lin et al., 2009; Vandenbussche et al., 2012; Van de Poel and Van Der Straeten, 2014). The lack of Cd-induced ethylene production in the double knock-out (KO) *acs2-1acs6-1* mutants highly diminished the fast-induced ethylene response observed in wildtypes (Schellingen et al., 2014).

Exposure to abiotic stressors, such as Cd, also leads to an oxidative challenge at the cellular level inducing both damaging and protective signalling pathways (Cuypers et al., 2012). Cadmium is known to increase the levels of reactive oxygen species (ROS) for example through alterations in expression and activation of ROS producing enzymes (Cuypers et al., 2011; Remans et al., 2010; Sharma and Dietz, 2009). To control ROS production and maintain the cellular redox homeostasis within its physiological limits, plants have developed an antioxidative defence system consisting of enzymes, e.g. superoxide dismutase, catalase as well as metabolic components, e.g. glutathione (GSH), ascorbate (AsA) (Cuypers et al., 2009; Keunen et al., 2013). The antioxidative metabolite GSH is known to be affected by Cd stress and plays an important role in Cd chelation as well as in the control of the oxidative challenge (Jozefczak et al., 2014). Ethylene is known to mediate ROS production under different stress conditions (Mersmann et al., 2010; Montero-Palmero et al., 2014). Moreover, increasing evidence for a link between ethylene and the antioxidative metabolite

GSH is emerging. Yoshida et al. (2009) suggested that ethylene and salicylic acid protect against ozone-induced damage in *A. thaliana* leaves by increasing GSH biosynthesis. Cao et al. (2009) states that ethylene signalling mediates lead resistance in *A. thaliana* seedlings partially in a GSH dependent mechanism. Previous research evinced a transient induction of ethylene responses after short-term exposure of *Medicago sativa* to mercury (Hg) or *A. thaliana* to Cd (Montero-Palmero et al., 2014; Schellingen et al., 2014). However, the short-term influence of this transient ethylene production on oxidative stress parameters, and the consequent long-term influence on plant acclimation, remains to be established. We hypothesise that the transient peak of Cd-induced ethylene production influences oxidative stress and short-term responses, as well as long-term acclimation in *A. thaliana* seedlings. Therefore, we used the *acs2-1acs6-1* double KO mutant plants that previously showed an attenuated ethylene peak after acute Cd exposure, and studied short-term (24 & 72 h) responses at the molecular and metabolic level, and plant growth and reproduction capacity during prolonged exposure to sublethal Cd concentrations.

4.2 Methods

4.2.1 Plant material, culture, treatment and sampling

Arabidopsis thaliana (Columbia ecotype) wild-type (WT) and *acs2-1acs6-1* double KO-mutant seeds (N16581) were obtained from the European *Arabidopsis* Stock Centre (NASC). These mutant plants were described by Tsuchisaka et al. (2009) and they were checked for homozygosity of the double mutation by PCR.

Seeds were surface sterilised during 1 minute in 0.1% NaClO and afterwards thoroughly washed in sterile water. Subsequently, the seedlings were cultivated either (1) using a modified Hoagland nutrient solution on hydroponics according to Smeets et al. (2008) but using purified sand, or (2) on 12 x 12 cm vertical plates containing 50x diluted Gamborg's B5 macro- and micronutrients according to Remans et al. (2012). Established growth conditions for both culturing systems were 12 h photoperiod with day/night temperatures of respectively 22/18 °C and 65 % relative humidity. A combination of blue, red and far-red led modules (Philips Green-Power LED modules, the Netherlands) was used to simulate the photosynthetic active radiation (PAR) spectrum of sunlight with a photosynthetic photon flux density of 170 µmol m^{-1} s^{-1} at the leaf level (Keunen et al., 2011).

Three-week-old plants grown on hydroponics were exposed to 5 or 10 µM $CdSO_4$ at the root level (except for control plants). These sublethal concentrations are commonly found in the pore water of moderately contaminated soils (Krznaric et al., 2009) and were also applied in previous hydroponic growth experiments. The nutrient solution was refreshed twice a week. After 24 or 72 h of exposure, whole root and shoot systems were separated, sampled and snap frozen in liquid nitrogen prior to storage at -70 °C except for quantification of Cd contents and prolonged growth experiments. Biological replicates for each measured parameter (number of replicates displayed in table and figure captions) were sampled from various pots of the same conditions to avoid within pot correlation (Smeets et al., 2008). In the long-term experiment, 3-week-old plants were continuously exposed to Cd or grown under control conditions during the entire leaf developmental stage (until 40 days) and the number of leaves and rosette diameter were measured daily. Subsequently, the plants continued to grow

chronically exposed to Cd or under control conditions for 20 more days. Afterwards they were set to dry and the seeds were harvested per plant.

After sowing, the vertical agar plates (VAPs) were incubated at 4 °C for 2–3 days in the dark. Subsequently these germination plates were placed vertically in a culture room. After 7 days of growth, plants with approximately identical primary root lengths were transferred to treatment plates, for which appropriate amounts of concentrated filter-sterilized $CdSO_4$ was added into the medium. A range of 0-10 µM $CdSO_4$ was used according to previous VAP experiments (Remans et al., 2012). In all treatment plates 1 cm of agar was removed at the top to create an air gap for the shoots. After another 7 days of growth on VAPs after transfer, plates were scanned on an Epson v330 Photo (Epson, Japan) and root growth was analysed using the Optimas 6.1 Image analysis program (Media Cybernetics, USA).

4.2.2 Quantification of Cd contents

Roots and leaves of hydroponically grown plants were harvested. Roots were washed for 15 min with ice-cold 10 mM $Pb(NO_3)_2$ and rinsed in distilled water at 4 °C to exchange surface-bound elements (Cuypers et al., 2002). Leaves were rinsed with distilled water. Samples were oven-dried at 80 °C and digested in HNO_3 (70-71 %) in a heat block. Cadmium concentrations in the extracts were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Perkin-Elmer, 1100B, USA). As references, blanks (HNO_3 only) and certified standard samples (NIST Spinach (1570a)) were analysed.

4.2.3 Gene expression analysis

RNA was extracted using the RNAqueous® Total RNA Isolation Kit (Life Technologies, Belgium), according to the manufacturer's instructions, from frozen root and leaf tissues of hydroponically grown plants. The samples were disrupted under frozen conditions in 2mL microcentrifuge tubes using two stainless steel beads and the Retsch Mixer Mill MM 400 (Retsch, Belgium). RNA concentration and purity were evaluated spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, USA). DNase treatment with the TURBO DNA-free™ Kit (Life Technologies) was performed to eliminate possible genomic DNA contamination. For each sample, one µg of the treated RNA was converted

to single stranded cDNA using the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Bio Inc., the Netherlands) according to the manufacturer's instructions. The cDNA was diluted 10-fold in 1/10 diluted TE buffer (1 mM Tris–HCl, 0.1 mM Na_2-EDTA, pH 8.0; Sigma–Aldrich, Belgium) and stored at -20 °C. Quantitative real-time PCR was performed in an optical 96-well plate with the 7900HT Fast Real-Time PCR System (Life Technologies) using SYBR Green chemistry. Gene-specific forward and reverse primers were designed via the Primer Express software (v2.0, Life Technologies). Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C) followed by the generation of a dissociation curve to verify amplification specificity. Reactions contained 2 µL diluted cDNA template (or RNase-free H_2O for the 'no template controls'), 5 µL 2x Fast SYBR® Green Master Mix (Life Technologies), forward and reverse primers (300 nM each, unless otherwise mentioned in Supplemental file 4.1) in a total volume of 10 µL. The specificity of the used primer pairs was checked *in silico* using Blast (http://www.arabidopsis.org /Blast/index.jsp) and after qPCR by verifying single peaks on the dissociation curve. In addition, primer efficiency (E) was evaluated on a standard curve generated using a twofold dilution series of a mixed sample over at least five dilution points and verified to be higher than 80 % (E = 10^(-1/slope)). In supplemental file 4.1, all gene annotations, primer sequences and primer efficiencies are shown. Gene expression levels were calculated according to the $2^{-\Delta Cq}$ method relative to the sample with the highest expression (minimum Cq). The data obtained were normalised using the geometric average of the $2^{-\Delta Cq}$ values of three stable reference genes selected out of a set of 10 (Remans et al., 2008) by geNorm (v3.5) and Normfinder (v0.953) algorithms (Andersen et al., 2004; Vandesompele et al., 2002). The most stable reference genes were used to determine sample-specific normalisation factors (Supplemental file 4.1). Supplemental file 4.2 shows the RT-qPCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

4.2.4 Glutathione content

The oxidised and reduced forms of glutathione were extracted and spectrophotometrically measured according to the plate reader method previously described by Queval and Noctor (2007) and modified by Jozefczak et al. (2014). Frozen root and leaf samples (100 mg) were ground in liquid nitrogen using a cooled mortar and pestle and further homogenised by adding 200 mM HCl (800 µl per 85 mg (roots) or 120 mg (leaves) fresh weight). After centrifugation (10 min, 16 000g, 4 °C), the pH of the samples was adjusted to 4.5. Unless otherwise mentioned, the samples were kept at 4 °C during the entire procedure. The spectrophotometric measurement of GSH and GSSG is monitored at 412 nm during 5 min and is based on the reduction of 5,5-dithiobis(2-nitro-benzoic acid) (DTNB, 600 µM) by the action of glutathione reductase (GR, 1U mL^{-1}) in the presence of NADPH (500 µM). Total glutathione (reduced and oxidised) concentrations were calculated relative to a standard curve ranging from 0 to 125 pmol GSH for roots and 0 to 500 pmol GSH for leaves. The oxidised GSSG concentration was measured by incubating the samples with 2-vinyl-pyridine (2-VP, 1% v/v) during 30 min at room temperature to precipitate all free reduced GSH present in the sample. Prior to the measurement, 2-VP was precipitated by centrifuging the samples twice (10 min, 16000g, 4 °C). For quantification purposes, a GSSG standard curve ranging from 0 to 100 pmol for roots and leaves was incubated with 2-VP and measured in duplicate concurrently with the samples. By subtracting the concentration of oxidised GSSG from the total glutathione concentration, the amount of reduced GSH was calculated (Queval and Noctor, 2007).

4.2.5 Statistical analysis

Outliers were determined using the extreme studentised deviate analysis (GraphPad Software, USA) at significance level 0.05. The datasets were analysed via the linear model procedure in R (R Development Core Team, 2012). Both normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) were checked; transformations were applied when necessary to approximate normality. For gene expression data, normalised relative quantities were log transformed prior to further statistical analysis. Normally distributed data were analysed using the one- or two-way ANOVA procedure. Tukey–Kramer

adjustment for multiple comparisons was applied to obtain corrected p-values. The statistical analyses of non-normally distributed data were based on the non-parametric Kruskal–Wallis test followed by the post-hoc pairwise Wilcoxon rank sum test. Figure captions indicate the experiment specific statistical analysis.

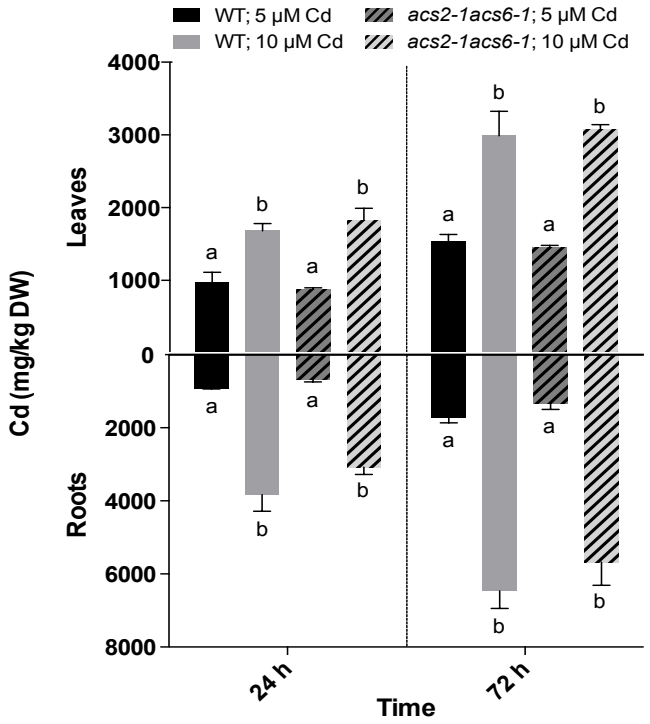
4.3 Results

Previous research showed that 24 and 72 h of exposure to Cd induces the biosynthesis of ethylene in wild-type (WT) *A. thaliana* plants but not in the *acs2-1acs6-1* double KO-mutants (Schellingen *et al*. 2014). Here, short-term growth responses (24 and 72 h) in roots and leaves of Cd-exposed WT and *acs2-1acs6-1* mutant plants exposed to sublethal Cd concentrations (5 & 10 µM Cd) were studied and compared to long-term responses. In addition, the Cd-induced oxidative challenge was measured after short-term exposure at molecular and biochemical levels.

4.3.1 Cadmium content and plant growth

The internal Cd concentration increased in function of the externally applied Cd concentration in roots and leaves of both WT and *acs2-1acs6-1* mutant plants (Fig. 4.1). No significant differences in Cd accumulation were observed between both genotypes (Fig. 4.1). The translocation factor (Cd concentration in leaves / Cd concentration in roots) also showed no significant differences between both genotypes. A significantly decreased translocation after exposure to 10 µM Cd was noticed, resulting in an internal Cd concentration that was approximately twice as high in roots as compared to leaves (Table in Fig. 4.1).

Root fresh weight of WT and *acs2-1acs6-1* mutant plants was not significantly affected after 24 h exposure, but was decreased after 72 h of exposure to 5 or 10 µM Cd (Table in Fig. 4.1). Leaf fresh weight of both genotypes was not affected after 24 h Cd exposure, however 72 h exposure to 10 µM Cd led to a significant decrease of leaf fresh weight in WT plants, whereas this was not significant in *acs2-1acs6-1* mutants (Table in Fig. 4.1). Although, no significant difference was observed in leaf fresh weight between control and 5 µM Cd-exposed plants after 72 h, the leaf fresh weight of *acs2-1acs6-1* mutants was significantly higher than of WT plants exposed to 5 µM Cd (Table in Fig. 4.1).



			24 h		72 h	
			wild type	*acs2-1acs6-1*	wild type	*acs2-1acs6-1*
FW (mg)	*Leaves*	0 µM $CdSO_4$	55.4 ± 1.5a	52.7 ± 3.4a	91.4 ± 6.8ac	94.8 ± 6.8c
		5 µM $CdSO_4$	48.2 ± 2.6a	51.3 ± 2.5a	71.8 ± 6.6ab	91.1 ± 7.2c
		10 µM $CdSO_4$	47.5 ± 2.8a	51.4 ± 4.3a	59.8 ± 4.4b	70.7 ± 2.3bc
	Roots	0 µM $CdSO_4$	20.5 ± 0.4a	17.6 ± 1.1a	40.7 ± 2.0a	38.5 ± 2.7a
		5 µM $CdSO_4$	18.6 ± 1.7a	16.9 ± 0.9a	25.6 ± 1.6b	28.8 ± 1.7b
		10 µM $CdSO_4$	17.7 ± 0.7a	16.1 ± 1.9a	21.4 ± 2.2b	21.6 ± 1.8b
Trans Fac		5 µM $CdSO_4$	1.1 ± 0.1a	1.3 ± 0.2a	0.9 ± 0.1a	1.1 ± 0.2a
		10 µM $CdSO_4$	0.5 ± 0.1b	0.6 ± 0.1b	0.5 ± 0.1b	0.6 ± 0.1b

Figure 4.1 Cd content and plant fresh weight. *A comparison of the Cd content (mg kg^{-1} DW^{-1}), fresh weight (mg FW) and translocation factor (Trans Fac) in roots and leaves of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data represent mean ± s.e. of three to six biological replicates. The letters a-c represent groups with a significantly different Cd content, fresh weight or translocation factor after treatment (Tukey's test: $p < 0.05$). Statistics was performed separately for Cd concentrations, fresh weight and translocation factor within each exposure time and within each organ.*

Since Cd-induced effects on growth became visible after 72 h, and given the differential effect of the mutation on leaf fresh weight under Cd exposure, we studied differences in root and leaf growth as well as reproductive capacity after even more prolonged exposure of WT and *acs2-1acs6-1* mutant plants to Cd in vertical agar plates and in hydroponics. When 7-day-old plants grown on vertical agar control plates were transferred during 7 days to plates containing a range of Cd concentrations, the primary and total lateral root length decreased in a dose-dependent manner (Fig. 4.2 A & B). However, no significant differences could be observed in the root growth between WT and *acs2-1acs6-1* mutant plants (Fig. 4.2 A & B).

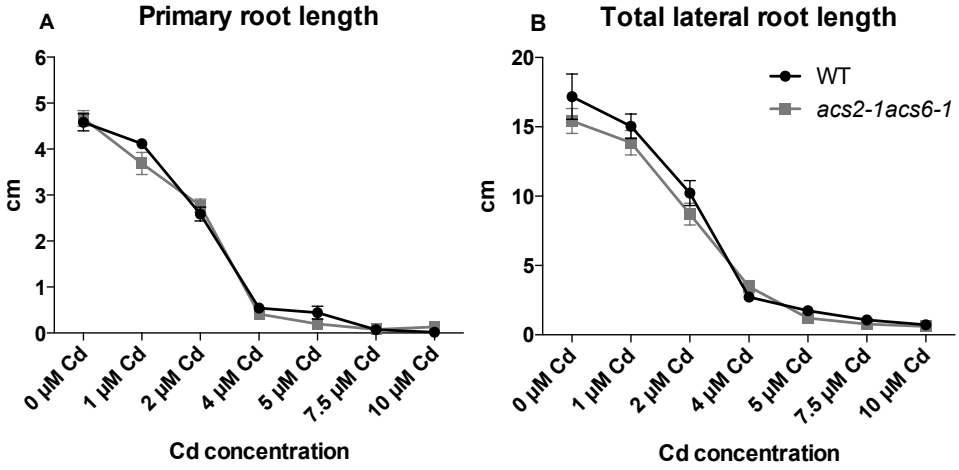


Figure 4.2 Root growth in vertical agar plates. *A comparison of the primary root length (A) and the total lateral root length (B) of wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana seedlings exposed to different concentrations of $CdSO_4$ or grown under control conditions during 7 days on vertical agar plates. Data represent mean ± s.e. of twelve biological replicates. Statistics was performed separately for each Cd concentration (Tukey's test: $p < 0.05$).*

In the hydroponic experimental set-up, 19-day-old plants were exposed to 5 and 10 µM Cd and plant growth was followed during 3 weeks. Rosette diameter as well as the number of leaves per rosette (Fig. 4.3 A & B) decreased in both WT and *acs2-1acs6-1* mutant plants. Contrary to the 72 h exposure to Cd, no significant differences could be observed in leaf growth between both genotypes (Fig. 4.3 A & B).

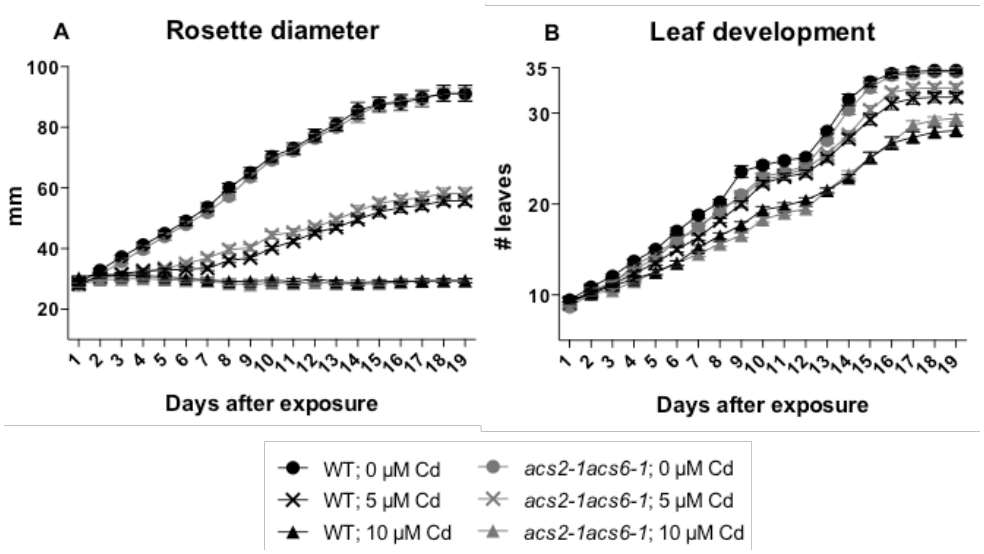


Figure 4.3 Rosette growth. *A comparison of the rosette diameter (A) and leaf growth (B) of 40-day-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed during the last 19 days of the experiment to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data represent mean ± s.e. of fifteen biological replicates. Statistics was performed separately for each day between the WT and mutant within the same condition (Tukey's test: $p < 0.05$).*

All chronically exposed plants produced seeds with normal germination capacity (Fig. 4.4). The average number of seeds per plant significantly decreased in both genotypes, but was never significantly different between the WT and *acs2-1acs6-1* mutant plants for both Cd concentrations (Fig. 4.4).

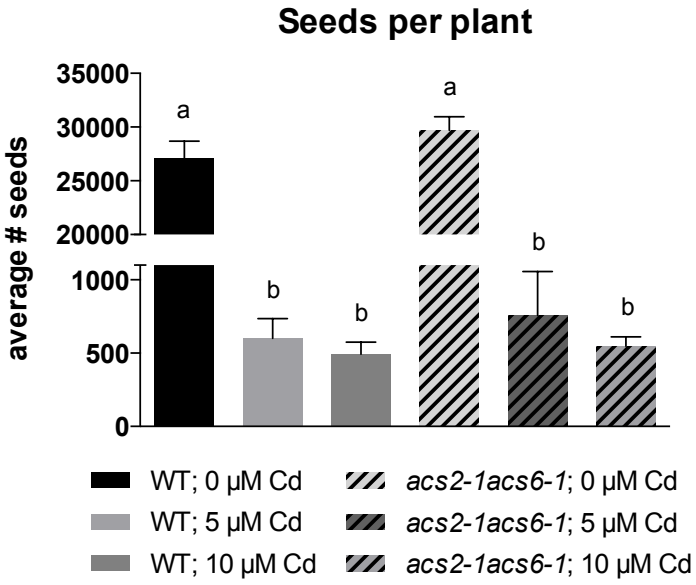


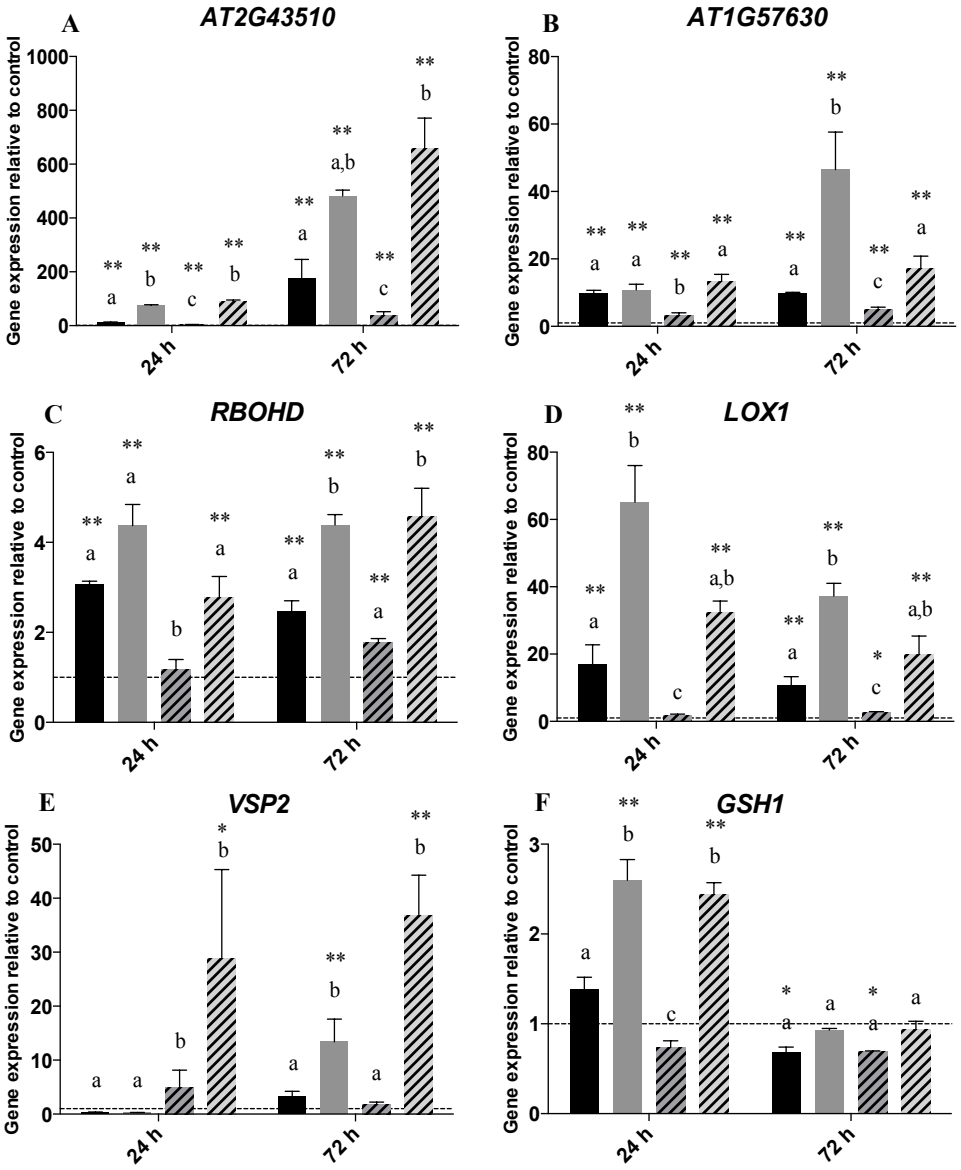
Figure 4.4 Seeds per plant. *A comparison of the number of seeds per plant of 60-day-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed during the last 39 days of the experiment to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data represent mean ± s.e. of fifteen biological replicates (Tukey's test: $p < 0.05$).*

4.3.2 Cadmium-induced oxidative challenge

Since ethylene is known to play a central role in the responses to different types of abiotic stresses and is capable of mediating the production of ROS, we investigated whether and how the ethylene biosynthesis affects the Cd-induced oxidative challenge by using WT and double KO *acs2-1acs6-1 A. thaliana* plants (Han et al., 2013; Mersmann et al., 2010).

4.3.2.1 Cadmium-induced effects on the oxidative stress hallmark genes

Gadjev et al. (2006) described a set of 5 genes, referred to as hallmark genes for general oxidative stress. The transcript levels of these genes were upregulated more than 5-fold in several experiments eliciting oxidative stress, independent of the type and production site of the ROS. In roots of WT and *acs2-1acs6-1* mutant plants, most of the transcript levels of these 5 oxidative stress marker genes were upregulated after 24 or 72 h exposure to 5 or 10 µM Cd (Supplemental file 4.3, Fig. 4.5 A & B). However, it should be noted that the transcript levels in *acs2-1acs6-1* mutant plants were in most cases significantly lower as compared to those of WT plants after exposure to 5 µM Cd, whereas exposure to 10 µM Cd induced similar increases in gene expression levels in WT and *acs2-1acs6-1* mutant plants (Supplemental file 4.3, Fig. 4.5 A & B). Also in the leaves, the *acs2-1acs6-1* mutant plants generally showed a lower expression of the 5 marker genes as compared to the WT (Supplemental file 4.4, Fig. 4.6 A & B). After 24 h of exposure to 5 or 10 µM Cd, the transcript levels of the 5 oxidative stress marker genes significantly increased in leaves of both genotypes. The expression of these genes was always significantly higher in WT plants compared to the *acs2-1acs6-1* mutants after exposure to 5 µM Cd. This could also be observed after exposure to 10 µM Cd except for the expression of *AT2G21640* and *AT1G05340* (Supplemental file 4.4, Fig. 4.6 A & B). After 72 h of exposure to 5 µM Cd, only the expression of *AT2G43510* was significantly lower in the *acs2-1acs6-1* mutant plants compared to the WT, while exposure to 10 µM Cd did not induce significant differences between both genotypes (Supplemental file 4.4, Fig. 4.6 A & B).


A
AT2G43510
B
AT1G57630
C
RBOHD
D
LOX1
E
VSP2
F
GSH1
Gene expression relative to control
24 h
72 h

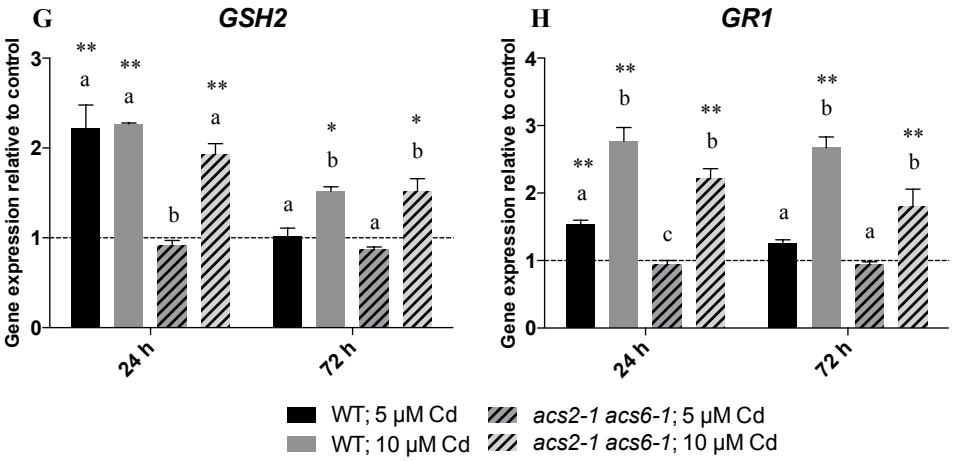
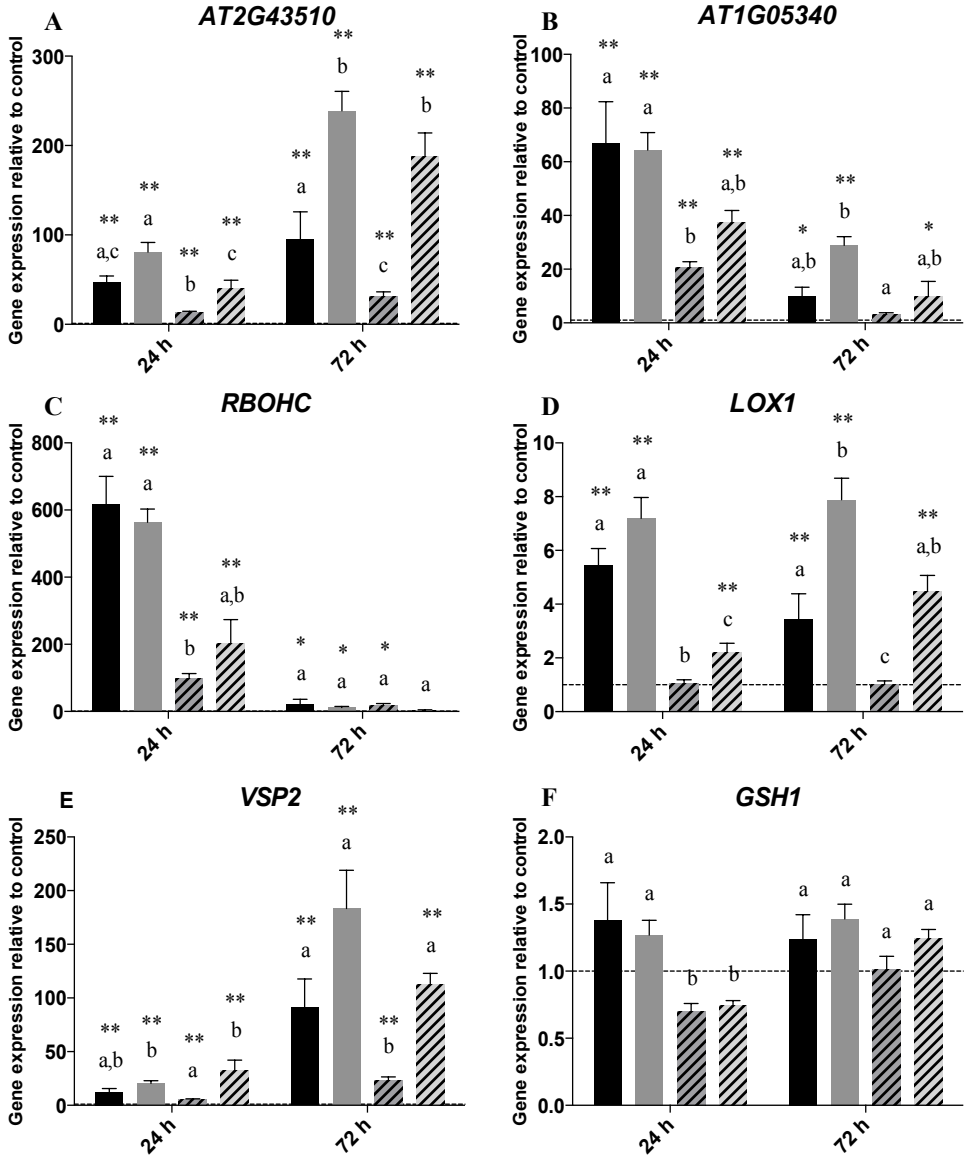


Figure 4.5 Relative expression of oxidative stress related genes in roots.

*A comparison of the relative expression of two oxidative stress hallmark genes (A & B), RBOHD (C), LOX1 (D), VSP2 (E), GSH1 (F), GSH2 (G) and GR1 (H) in roots of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Per time point, data show mean ± s.e. of at least 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks (Tukey's test: * $p < 0.05$, ** $p < 0.01$). The letters a-c represent groups with a significantly different gene expression between both genotypes (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time.*


A
AT2G43510
B
AT1G05340
C
RBOHC
D
LOX1
E
VSP2
F
GSH1
Gene expression relative to control
24 h
72 h

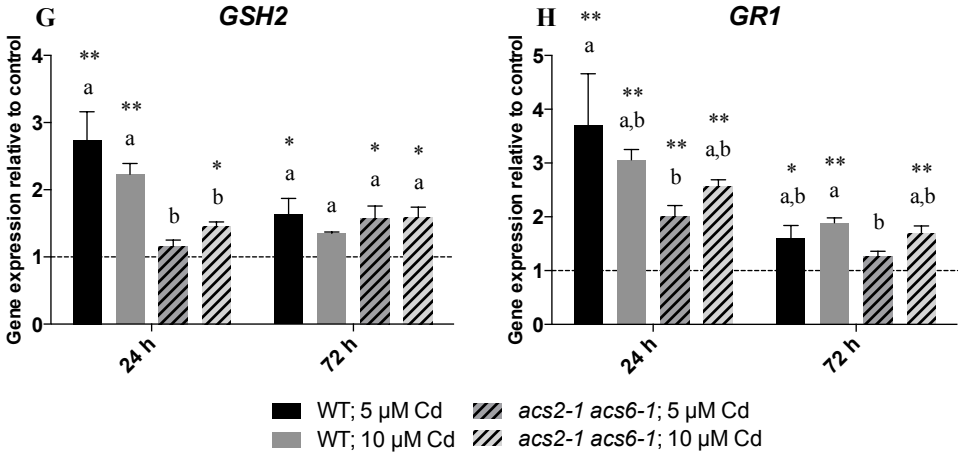


Figure 4.6 Relative expression of oxidative stress related genes in leaves.

*A comparison of the relative expression of two oxidative stress hallmark genes (A & B), RBOHC (C), LOX1 (D), VSP2 (E), GSH1 (F), GSH2 (G) and GR1 (H) in leaves of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Per time point, data show mean ± s.e. of at least 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks (Tukey's test: * $p < 0.05$, ** $p < 0.01$). The letters a-c represent groups with a significantly different gene expression between both genotypes (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time.*

4.3.2.2 Cadmium-induced effects on the expression of pro-oxidative and oxylipin-related genes

The effect of Cd exposure on a selection of pro-oxidative and oxylipin-related genes, based on their strong response to Cd exposure in previous experiments (Cuypers et al., 2011; Keunen et al., 2013; Remans et al., 2010), was investigated in roots and leaves of WT and *acs2-1acs6-1* mutant plants (Supplemental file 4.3 & 4.4, Fig. 4.5 & 4.6). The Cd-induced effect on different isoforms of the respiratory burst oxidase homologue (RBOH) genes, coding for a superoxide ($O_2^{-\circ}$) producing pro-oxidative enzyme, was studied. In roots, whereas no effect on *RBOHC* transcript levels was noticed after Cd exposure in both genotypes, the expression of *RBOHD* was significantly lower in the *acs2-1acs6-1* mutants as compared to the WT plants after 24 h exposure to 5 µM Cd (Fig. 4.5 C). In contrast to the WT plants, the transcript levels of *RBOHF* did not increase in the *acs2-1acs6-1* mutants after 24 h exposure to 5 µM Cd. Prolonged exposure (72 h) to both 5 & 10 µM Cd resulted in a significantly lower expression of *RBOHF* in the *acs2-1acs6-1* mutants compared to the WT plants (Supplemental file 4.3). Lipoxygenases (LOXs) have also been implicated in oxidative stress effects after exposure to toxic metals, potentially initiating lipid peroxidation. The products of LOX activity and subsequent reactions can also generate oxylipins, such as jasmonates, with a possible role as signalling molecules under metal stress (Maksymiec and Krupa, 2006). In contrast to WT plants, exposure to 5 µM Cd did not increase the expression of *LOX1* after 24 h in roots of *acs2-1acs6-1* mutant plants. After 72 h exposure to 5 µM Cd, the transcript levels of *LOX1* were induced in *acs2-1acs6-1* mutant plants but still significantly lower compared to the WT. Exposure to 10 µM Cd increased the expression of *LOX1* to a similar extent in both genotypes after 24 and 72 h (Supplemental file 4.3, Fig. 4.5 D). This response was also observed for the expression of *LOX6* (Supplemental file 4.3). The transcript levels of the vegetative storage protein2 (*VSP2*), a jasmonate responsive gene, were significantly higher in roots of *acs2-1acs6-1* mutant plants after 24 h exposure to Cd compared to WT plants (Stotz et al., 2011). After 72 h Cd exposure, no significant differences in *VSP2* expression were observed between both genotypes, although a nearly two-fold increase was visible in the *acs2-1acs6-1* mutant plants after exposure to 10 µM Cd (Supplemental file 4.3, Fig. 4.5 E).

Cadmium exposure caused an increased pro-oxidative gene expression in the leaves of WT plants. In the *acs2-1acs6-1* mutants this increase was much lower or even absent (Supplemental file 4.4). The transcript levels of the *RBOHC* gene were significantly higher in the WT compared to the *acs2-1acs6-1* mutant plants after 24 h exposure to 5 µM Cd. In contrast to the *acs2-1acs6-1* mutant plants, the expression of *RBOHC* was still upregulated after 72 h exposure to 10 µM Cd in WT plants (Supplemental file 4.4, Fig. 4.6 C). The expression of *RBOHD* was significantly lower in the *acs2-1acs6-1* mutants compared to the WT plants after 72 h exposure to 5 µM Cd. The transcript levels of *RBOHF* were significantly upregulated after 24 and 72 h exposure to 5 µM Cd and after 72 h exposure to 10 µM Cd in the WT plants. In the *acs2-1acs6-1* mutant plants, this upregulation was absent (Supplemental file 4.4). The transcript levels of the *LOX1* gene in leaves responded to Cd exposure in a similar way as in roots. The expression of LOX1 was significantly lower in the *acs2-1acs6-1* mutant plants compared to the WT after 24 h or 72 h exposure to 5 µM Cd (Supplemental file 4.4, Fig. 4.6 D). Exposure to 10 µM Cd increased the transcript levels of *LOX1* in both genotypes, although still significantly lower in the *acs2-1acs6-1* mutant plants after 24 h. The expression of *LOX2* always increased significantly in both WT and *acs2-1acs6-1* mutant plants after Cd exposure, without significant differences between both genotypes (Supplemental file 4.4). The transcript levels of *VSP2* were also always upregulated in both genotypes after Cd exposure. However, after 72 h exposure to 5 µM Cd, a significantly lower expression of *VSP2* was observed in the *acs2-1acs6-1* mutant plants (Supplemental table 4.4, Fig. 4.6 E).

4.3.2.3 Cadmium-induced effects on the glutathione metabolism

Cadmium exposure alters the transcript levels of genes encoding different enzymes involved in ROS scavenging and the GSH metabolism in WT *A. thaliana* plants (Cuypers et al., 2011; Jozefczak et al., 2014). Therefore, antioxidative gene expression as well as gene expression related to GSH metabolism and levels of the metabolite GSH were compared between WT and *acs2-1acs6-1* mutant plants to determine whether ethylene biosynthesis modifies Cd-induced antioxidant responses (Supplemental file 4.3 & 4.4, Fig. 4.5 & 4.6).

Genes encoding the enzymes involved in GSH synthesis (*GSH1* and *GSH2*) and GSH reduction (*GR1* and *GR2*) showed significant upregulation in roots of WT plants particularly after 24 h exposure to 10 µM Cd. In the *acs2-1acs6-1* mutant plants, the same could be observed for *GSH1*, *GSH2* and *GR1*. After exposure to 5 µM Cd however, *GSH2* and *GR1* were only significantly upregulated in WT plants but not in the *acs2-1acs6-1* mutants (Supplemental file 4.3, Fig. 4.5 F, G & H). After 72 h exposure, no significant differences between both genotypes were observed with decreased *GSH1* expression after 5 µM Cd exposure and increased transcript levels for *GSH2* and *GR1* after 10 µM Cd exposure (Fig. 4.5 F, G & H).

In the leaves, 24 h exposure to Cd did not significantly affect *GSH1* transcript levels, nevertheless they were lower in Cd-exposed *acs2-1acs6-1* mutants as compared to WT plants. The expression of *GSH2* was significantly upregulated after exposure to 5 or 10 µM Cd in both genotypes, but also significantly lower in the mutant plants (Fig. 4.6 F & G). The expression of *GR1* significantly increased while the transcript levels of *GR2* decreased in both genotypes. The transcript levels of *GR1* were significantly lower in the *acs2-1acs6-1* mutant plants compared to the WT after exposure to 5 µM Cd (Fig. 4.6 H). Prolonged exposure (72 h) to Cd diminished most responses on the expression of most GSH-related genes, without any significant difference between both genotypes (Fig. 4.6 F, G & H).

The Cd-induced effects at the metabolic level showed no significant differences between the WT and *acs2-1acs6-1* mutant roots in reduced (GSH), oxidised (GSSG) or total GSH content (Table 4.1). A significant increase was measured only for GSSG after exposure to 10 µM Cd for 24 h in both genotypes (Table 4.1). In leaves, Cd exposure had more pronounced effects on GSH content as compared to the roots (Table 4.1). After 24 h exposure, total and reduced GSH contents were maintained in WT plants, while in *acs2-1acs6-1* mutant plants GSH significantly decreased after exposure to 5 µM Cd (Table 4.1). After 72 h exposure to 10 µM Cd, total and reduced GSH levels were significantly increased in both genotypes without significant differences between the genotypes (Table 4.1).

Table 4.1. *Glutathione content (reduced, oxidised and total; nmol GSH equivalents g^{-1} FW) in roots and leaves of 3-week-old wild-type or acs2-1acs6-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 or 10 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data are given as mean ± s.e. of at least 4 biological replicates. The letters a-c represent groups with significantly different glutathione levels (Tukey's test: $p < 0.05$). Statistics were performed separately for reduced, oxidised and total glutathione within each time point. nd: levels below detection limit.*

		24 h		72 h	
Roots		**WT**	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
Total GSH+GSSG	**Control**	149.34 ± 7.86a	153.27 ± 10.83a	145.93 ± 11.62a	150.80 ± 18.12a
	5 µM Cd	141.60 ± 3.82a	125.19 ± 6.39a	145.00 ± 11.60a	126.03 ± 10.79a
	10 µM Cd	148.05 ± 12.58a	119.10 ± 10.05a	154.09 ± 5.10a	150.09 ± 2.42a
GSH	**Control**	148.02 ± 7.26a	153.19 ± 10.32a	146.47 ± 11.94a	145.26 ± 19.29a
	5 µM Cd	141.12 ± 2.73a	124.43 ± 7.88a	144.35 ± 13.48a	122.89 ± 10.72a
	10 µM Cd	143.23 ± 12.51a	114.34 ± 10.29a	146.30 ± 5.81a	140.45 ± 3.51a
GSSG	**Control**	1.33 ± 0.84a	nd	nd	5.54 ± 1.43a
	5 µM Cd	0.47 ± 1.10a	0.76 ± 1.52ab	0.65 ± 2.02a	3.14 ± 0.81a
	10 µM Cd	4.82 ± 0.98b	4.77 ± 0.62b	7.79 ± 2.19a	9.63 ± 2.88a
Leaves		**WT**	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
Total GSH+GSSG	**Control**	239.33 ± 9.72ab	251.91 ± 6.02ab	226.99 ± 17.22a	233.21 ± 6.85a
	5 µM Cd	229.33 ± 12.48ab	178.53 ± 13.87c	305.75 ± 9.60a	253.26 ± 18.92a
	10 µM Cd	262.86 ± 16.41a	211.38 ± 4.45bc	439.61 ± 39.97b	452.14 ± 42.26b
GSH	**Control**	229.31 ± 10.66a	235.29 ± 8.13a	217.83 ± 17.78a	218.01 ± 7.73a
	5 µM Cd	226.39 ± 11.69a	175.42 ± 14.19b	296.49 ± 7.67a	248.22 ± 17.57a
	10 µM Cd	256.07 ± 16.95a	206.61 ± 5.60ab	422.43 ± 39.92b	440.81 ± 39.92b
GSSG	**Control**	10.02 ± 1.10ac	16.62 ± 2.67c	9.16 ± 1.54ab	15.20 ± 1.07ab
	5 µM Cd	2.95 ± 0.61b	3.10 ± 0.66b	9.26 ± 2.18ab	5.04 ± 1.47b
	10 µM Cd	5.85 ± 0.17ab	4.78 ± 1.89ab	17.18 ± 4.10a	11.33 ± 2.37ab

4.4 Discussion

The stress hormone ethylene is known to be crucial for the survival of adverse environmental stimuli, both biotic and abiotic (Bartoli et al., 2013; Vandenbussche et al., 2012). Recently, it was evidenced that exposure to the toxic metal Cd rapidly induces the biosynthesis of ACC and ethylene mainly via an increased expression of *ACS2* and *ACS6* (Schellingen et al., 2014). In this study, we investigated the significance of this Cd-induced peak in ethylene production during moderate (5 µM) and more severe (10 µM) Cd stress using *acs2-1acs6-1* KO-mutant plants in which this ethylene response was attenuated (Schellingen et al., 2014).

4.4.1 Is ethylene biosynthesis required for long-term acclimation to Cd stress?

Cadmium accumulation was similar in the roots and leaves of both genotypes (Fig. 4.1). Differences in subsequently investigated parameters therefore arose from the lack of Cd-induced ethylene production rather than an altered Cd uptake.

Previous research in our group showed that the effects of Cd on vegetative growth of WT *A. thaliana* roots and leaves were dose-dependent while the generative growth was negatively influenced independent of the applied Cd concentration (Keunen et al., 2011; Remans et al., 2012). Furthermore, they observed that the vegetative plant growth of *A. thaliana* in hydroponic culture was only diminished after exposure to 5 or 10 µM Cd from 72 h onwards. In accordance with these results, we also observed a significant decrease in fresh weight of the roots of both investigated genotypes. In leaves however, *acs2-1acs6-1* mutant plants were clearly less sensitive to Cd at 72 h exposure in comparison with WT plants (Fig. 4.1). The fact that ethylene is a modulator of plant growth, negatively affecting cell elongation (Kieber et al., 1993; Rodrigues-Pousada et al., 1993), and known to promote Cd-induced senescence processes is a plausible explanation for this difference in leaf fresh weight (Yakimova et al., 2006). In the long-term experimental setup however, we observed similar effects in WT and the *acs2-1acs6-1* mutant plants (Fig. 4.2, 4.3 & 4.4). This indicates that both genotypes are equally sensitive to Cd in the long

term, which points towards an early and transient role for ethylene in the response to Cd stress.

Accordingly, the subsequent experiments focussed on the short-term Cd-induced oxidative challenge at the transcript and metabolic level in WT and *acs2-1acs6-1* mutants after exposure to moderate (5 µM Cd) or more severe (10 µM Cd) conditions.

4.4.2 Ethylene responses depend on Cd stress intensity

Kacperska (2004) already emphasised that stressors are recognised by different elements of the signal sensing system depending on the stress severity. The author proposed that the redox-mediated system is especially involved in the response to mild stresses and that the role of ROS in the mediation of stress responses depends on the severity of the stressor rather than the sensor type. Our measurements show that exposure to severe stress, *i.e.* 10 µM Cd, possibly activates multiple sensing and signalling systems, and in this way utilises compensatory mechanisms for the Cd-induced ethylene signal since most of the dissimilarities between WT and *acs2-1acs6-1* mutant plants could only be observed after moderate stress conditions.

Increased oxylipin signalling could imply such an ethylene compensatory mechanism as López et al. (2011) observed a link between 9-LOX-derived oxylipins and ethylene in the control of oxidative stress. Moreover, Mithöfer et al. (2004) showed that oxylipins can be involved in signalling following biotic and abiotic stresses. Lipoxygenases catalyse the first step in the biosynthesis of oxylipins and can be divided into 9- and 13-lipoxygenases, according to the position of oxygen incorporation in their substrates (Bannenberg et al., 2009). The 9-LOX enzyme LOX1, was shown to have a central role in Cd-induced stress responses (Keunen et al., 2013). The different effect of Cd exposure on *acs2-1acs6-1* mutant and WT plants supports the existence of a crosstalk between oxylipins and ethylene. Exposure to more severe concentrations of Cd (10 µM) significantly increased the expression of *LOX1* in the roots and leaves of *acs2-1acs6-1* mutants, diminishing most differences with the WT plants, especially in the roots (Fig. 4.5 D & 4.6 D). These results support a potential role for oxylipins as a signalling mechanism next to ethylene after exposure to severe Cd concentrations. Jasmonates are specific 13-LOX-derived oxylipins. In roots,

LOX6 was shown to be responsible for the stress-induced jasmonate accumulation independent from the leaves, despite the low expression of biosynthetic enzymes (Grebner et al., 2013). In agreement, only small increases in Cd-induced *LOX6* expression were observed in both genotypes (Supplemental file 4.3). Consequently, the expression of *VSP2*, a jasmonate responsive gene was measured. Short-term exposure to severe Cd concentrations significantly induced the expression of *VSP2* in the *acs2-1acs6-1* mutants but not in the WT, the latter confirming data by Lorenzo et al. (2003) (Fig. 4.5 E). These results suggest a potential role for jasmonates as a compensatory signal in roots. In leaves, LOX2 was shown to contribute the majority of jasmonate biosynthesis upon different stressors (Glauser et al., 2009; Seltmann et al., 2010). In accordance with our data, previous research in our group by Remans et al. (2010) and Cuypers et al. (2011) also showed that *LOX2* expression increased in the leaves of Cd-exposed WT *A. thaliana* plants. The expression of *LOX2* did not significantly differ between both genotypes (Supplemental file 4.4). Nevertheless, the expression of *VSP2* increased in *acs2-1acs6-1* mutant plants in a dose dependent way that was absent in the WT plants (Supplemental file 4.4, Fig. 4.6 E). These results again support a potential role for jasmonates as a compensatory signal in severely stressed *A. thaliana* leaves.

As most of the different responses between WT and *acs2-1acs6-1* mutant plants attenuate after exposure to 10 µM Cd, the remainder of the discussion focuses on differences evoked by moderate Cd stress, *i.e.* 5 µM Cd exposure.

4.4.3 Ethylene production is involved in the oxidative challenge under moderate Cd exposure

The Cd-induced oxidative challenge was investigated by measuring the expression of five oxidative marker genes in WT and the *acs2-1acs6-1* mutant plants. In the roots and leaves of *acs2-1acs6-1* mutant plants, significantly lower transcript levels of these oxidative stress marker genes were observed compared to the WT after 24 h Cd exposure (Supplemental file 4.3 & 4.4; Fig. 4.5 & 4.6 A & B;). In the leaves, prolonged exposure diminishes most of these differences, again supporting the transient character of ethylene responses (Supplemental file 4.4, Fig. 4.6 A & B). In accordance, Mersmann et al. (2010) also observed interplay between ethylene and ROS production in *A. thaliana*

exposed to virulent bacteria. To support this decrease in expression of the oxidative stress marker genes in *acs2-1acs6-1* mutant plants, we investigated the transcript levels of different pro-oxidative *RBOH* genes, ROS (superoxide) producing NADPH oxidases, which are clearly induced upon Cd exposure (Remans et al., 2010; Smeets et al., 2009). Indeed, in roots the transcript levels of *RBOHD* and *RBOHF* were upregulated in the WT plants, while this could not be observed in the *acs2-1acs6-1* mutant plants (Supplemental file 4.3, Fig. 4.5 C). In leaves, *RBOHC* was the predominant isoform significantly induced in WT plants. In the *acs2-1acs6-1* mutant plants the expression of *RBOHC* was significantly lower after 24 h exposure (Fig. 4.6 C). Ethylene is known to be an important activator of NADPH oxidases, leading to superoxide production and subsequently oxidative stress (Chae and Lee, 2001; Jakubowicz et al., 2010). Moreover, Montero-Palmero et al. (2014) proposed that the induction of an oxidative burst through NADPH oxidases in mercury-exposed (Hg) alfalfa is connected to ethylene. In summary, we can conclude that ethylene biosynthesis is indisputably involved in the early oxidative challenge, induced by moderate Cd exposure.

Plants respond to oxidative stress by activating their antioxidative defence mechanisms comprising ROS scavenging enzymes and metabolites (Cuypers et al., 2011). This study especially focussed on GSH, a key metabolite in Cd responses due to its chelating and antioxidant properties, and the gene expression of biosynthetic and recycling enzymes (Rauser, 2001; Jozefczak et al., 2014). Many differences in the GSH metabolism could be observed between WT and *acs2-1acs6-1* mutant plants after 24 h Cd exposure, while prolonged exposure (72 h) neutralises most differences (Fig. 4.5 & 4.6; Table 4.1). Whereas the significantly lower expression of GSH metabolic enzymes observed in the *acs2-1acs6-1* mutant plants did not result in significantly lower GSH levels in roots (Fig. 4.5 F, G & H; Table 4.1), it did in the leaves (Fig. 4.6 F, G & H; Table 4.1). Yoshida et al. (2009) also observed the necessity of *GSH2* and *GR1* expression in ozone-exposed *Arabidopsis thaliana* plants to maintain GSH levels. Although using a different experimental setup, Masood et al. (2012) also concluded that ethylene was necessary to maintain GSH in Cd-treated mustard plants. The decreased GSH levels in leaves of Cd-exposed *acs2-1acs6-1* mutants plants after 24 h (Table 4.1) again demonstrate the early involvement of the

ethylene response during Cd stress. Prolonged exposure to Cd diminishes the differences between both genotypes, pointing to other 'delayed responses' compensating this Cd-induced ethylene response. The upregulation of GSH biosynthesis in response to oxidative stress conditions like metal toxicity has already been established (Jozefczak et al., 2012). Therefore, a possible explanation for the early decrease in GSH biosynthesis in the leaves of the *acs2-1acs6-1* mutant plants is the reduced oxidative stress, shown by the significantly lower Cd-induced transcript levels of the pro-oxidative and oxidative stress marker genes in these mutant plants.

The following link between ethylene biosynthesis and oxidative stress after short-term exposure to moderate Cd concentrations is hypothesised. Cadmium is known to induce ethylene biosynthesis through the expression of *ACS2* and *ACS6* (Schellingen et al., 2014). This increase in ethylene production can eventually enhance ethylene signalling, eliciting signals that can further amplify localised bursts of ROS production through NADPH oxidases resulting in oxidative challenge. Besides oxidative damage, the increased ROS production can also activate signalling pathways that influence GSH metabolism. Exposure to a more severe (10 µM Cd) concentration of Cd diminishes the differences in response between the WT and *acs2-1acs6-1* mutant plants. This could be due to different compensatory mechanisms directly causing a large increase in ROS production and/or activating different stress-induced signals, overwhelming the plants signal sensing system and causing irreversible damage. Furthermore, unknown delayed compensatory mechanisms also cause this Cd-induced ethylene response to be transient, allowing acclimation to long-term Cd exposure.

The ethylene biosynthesis pathway is therefore suggested to play an important role in fine-tuning the 'early response' to moderate Cd stress in *A. thaliana*. Further research is necessary to identify potential candidate compensatory mechanisms, such as jasmonates. Last but not least, it is important to link the results of this study to the ethylene signal transduction pathway, in order to increase our understanding of the interaction between ethylene and Cd-induced oxidative stress.

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SUPPLEMENTAL FILES

Supplemental file 4.1. *Forward (FW) and reverse (REV) primers used to determine gene expression levels via quantitative real-time PCR. E-E-jn, exon-exon junction; SAND, SAND family; TIP41-like, tonoplast intrinsic protein 41-like; UBC, ubiquitin-conjugating enzyme; PPR, pentatricopeptide repeat; ACS, ACC synthase; ACO, ACC oxidase; ERF, ethylene response factor; ETR, ethylene receptor; PDF, plant defensin; VSP, vegetative storage protein; UPOX, upregulated by oxidative stress; TIR, Toll-Interleukin-1; LOX, lipoxygenase; RBOH, respiratory burst oxidase homologue; GSH1, glutamate-cysteine ligase; GSH2, glutathione synthetase; CSD, Cu/Zn superoxide dismutase; FSD, Fe superoxide dismutase; GR, glutathione reductase; MKK, mitogen-activated protein kinase kinase; MPK, mitogen-activated protein kinase. * ACS4, ACS5, AS7, ACS8 primer concentrations were increased to 900 nM. ** ERF1 primer concentrations were increased to 600 nM.* [1] *Root reference genes.* [2] *Leaf reference genes.*

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Reference genes					
AT2G28390	*SAND family*[1,2]	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13 and 14	61	97.41%
AT4G34270	*TIP41-like*[1,2]	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn and Exon 2	61	90.60%
AT5G25760	*UBC*[2]	FW: CTGCGACTCAGGGAATCTTCTAA REV: TTGTGCCATTGAATTGAACCC	E3-E4-jn and Exon 4	61	102.61%
AT5G55840	*PPR gene*[1]	FW: AAGACAGTGAAGGTGCAACCTTACT REV: AGTTTTTGAGTTGTATTTGTCAGAGAAAG	Exon 3	59	81.50%
Genes encoding ACC Synthase (ACS)					
AT2G22810	*ACS4**	FW: GTTACCAAGAACCCTCAAGGCA REV: TGTTTTGTGCAAGCCATGACTC	Exon 1 and 2	91	101.50%
AT5G65800	*ACS5**	FW: TTTTGCCTACTCCTTACTATCCTGGA REV: TTAGAGCTTGAGCAGTGAATGGG	E2-E3-jn and Exon 3	91	94.50%
AT4G26200	*ACS7**	FW: ACGACGCCCTTCTAGTTCCC REV: CAGTGGATGGGTACTATTTTCACTCC	Exon 2 and 3	91	101.50%
AT4G37770	*ACS8**	FW: GAAGGCCAATCCATATTTCGG REV: CCGACATGAAATCCGCCATA	Exon 2 and 3	91	101%
AT4G08040	*ACS11*	FW: AGATGCCTTTCTTATCCCTGCAC REV: GCAATGGATAGGAACAATCTCTACTCC	Exon 3 and 4	91	102.50%
Genes encoding ACC Oxidase (ACS)					
AT1G62380	*ACO2*	FW: TCTACGTTCGTCACCTCCCTCA REV: CTCTTACCAAAGTCTTTCATGGCC	Exon 2 and 3	91	100.76%
AT1G05010	*ACO4*	FW: CTCCGATGTCCCTGATCTCG REV: ATCCAGTAGCTCCTCCGACAACT	Exon 2 and 3	91	97.50%
Ethylene and jasmonate responsive genes					
AT3G23240	*ERF1***	FW: TCCTCGGCGATTCTCAATTTT REV: CAACCGGAGAACAACCATCCT	Exon 1	91	98.10%
AT4G17490	*ERF6*	FW: CCGGAGATTTTTGATTTCGATG REV: CAGTAACGCGAGGAGGATTCG	Exon 1	91	94.12%
AT3G23150	*ETR2*	FW: TTCGAACCGGGCAGTTACAC REV: AATGGCGGTAAGGCAATCG	Exon 2	91	87.50%
AT5G44420	*PDF1.2*	FW: TTTGCTGCTTTCGACGCAC REV: GCATGCATTACTGTTTCCGCA	E1-E2-jn and Exon 2	99	94.45%
AT5G24770	*VSP2*	FW: GGCGTGACCTACTGGAAGCA REV: CGAGACTCTTCCTCACCTTTGACTT	Exon 2 and 3	91	95.09%

AGI	Annotation	Primer sequences (5′-3′)	Exon location	Amplicon size (bp)	Primer efficiency
Genes encoding markers for ROS-induced gene expression					
AT2G21640	*UPOX*	FW: GACTTGTTTCAAAAACACCATGGAC REV: CACTTCCTTAGCCTCAATTTGCTTC	Exon 1 and 2	91	93.77%
AT2G43510	*Defensin-like*	FW: ATGGCAAAGGCTATCGTTTCC REV: CGTTACCTTGCGCTTCTATCTCC	Exon 1 and 2	91	98.42%
AT1G19020	*Unknown*	FW: GAAAATGGGACAAGGGTTAGACAAA REV: CCCAACGAAAACCAATAGCAGA	Exon 1	92	99.30%
AT1G05340	*Unknown*	FW: TCGGTAGCTCAGGGTAAAGTGG REV: CCAGGGCACAACAGCAACA	Exon 2 and 3	91	101.62%
AT1G57630	*TIR-class*	FW: ACTCAAACAGGCGATCAAAGGA REV: CACCAATTCGTCAAGACAACACC	Exon 1	91	94.56%
Genes encoding ROS producing enzymes					
AT1G55020	*LOX1*	FW: TTGGCTAAGGCTTTTGTCGG REV: GTGGCAATCACAAACGGTTC	Exon 6 and 7	101	99.13%
AT3G45140	*LOX2*	FW: TTTGCTCGCCAGACACTTG REV: GGGATCACCATAAACGGCC	Exon 3 and 4	102	86.65%
AT1G67560	*LOX6*	FW: GGCGATTTGACATGGAAGGA REV: ACAAGCCTCACGCCACATTC	Exon 8 and 9	91	108.00%
AT5G51060	*RBOHC*	FW: TCACCAGAGACTGGCACAATAAA REV: GATGCTCGACCTGAATGCTC	Exon 6 and 7	101	92.09%
AT5G47910	*RBOHD*	FW: AACTCTCCGCTGATTCCAACG REV: TGGTCAGCGAAGTCTTTAGATTCCT	Exon 1	91	93.96%
AT1G64060	*RBOHF*	FW: GGTGTCATGAACGAAGTTGCA REV: AATGAGAGCAGAACGAGCATCA	Exon 11 and 12	99	96.58%
Genes encoding antioxidative enzymes					
AT4G23100	*GSH1*	FW: CCCTGGTGAACTGCCTTCA REV: CATCAGCACCTCTCATCTCCA	Exon 5 and 6	101	98.60%
AT5G27380	*GSH2*	FW: GGACTCGTCGTTGGTGACAA REV: TCTGGGAATGCAGTTGGTAGC	Exon 11 and 12	101	92.60%
AT1G08830	*CSD1*	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5 and E6-E7-jn	102	87.52%
AT2G28190	*CSD2*	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6 and E7-E8-jn	101	98.56%
AT4G25100	*FSD1*	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4 and E6-E7-jn	101	92.76%
AT3G24170	*GR1*	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15 and 16	101	95.69%
AT3G54660	*GR2*	FW: TAGGGTTGGAGAATGTTGGCG REV: GCCCAGATGGATGGAACAGAT	Exon 5 and 6	91	99.82%
Genes encoding signal transduction genes					
AT1G73500	*MKK9*	FW: CGATACTTCACCGACGACAGG REV: GCGAGAGATGGAGATTCTCCG	Exon 1	91	97.51%
AT3G45640	*MPK3*	FW: GACGTTTGACCCCAACAGAA REV: TGGCTTTTGACAGATTGGCTC	Exon 5 and 6	103	100.37%
AT2G43790	*MPK6*	FW: TAAGTTCCCGACAGTGCATCC REV: GATGGGCCAATGCGTCTAA	Exon 5 and 6	101	107.68%
AT2G38470	*WRKY33*	FW: TCATCGATTGTCAGCAGAGACG REV: CCATTCCCACCATTTGTTTCAT	Exon 3 and 4	92	97.39%

***Supplemental file 4.2**. 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).*

Sample/Template	
Source	Leaves (entire rosette) of *Arabidopsis thaliana* plants cultivated in hydroponics
Method of preservation	Liquid nitrogen
Storage time	3 weeks at - 70 °C freezer
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 file 4.1
Amplicon details	Exon location and amplicon size: Supplemental file 4.1
Primer sequences	Supplementary file 4.1
In silico	Primers were blasted using the BLAST tool at http://arabidopsis.org/
Empirical	A primer concentration of 300 nM was used unless stated otherwise Annealing temperature: 60 °C
Priming conditions	Combination of oligodT-primers and random hexamers
PCR efficiency	Dilution series (slope, y-intercept and r^2; Supplemental file 4.1)
Linear dynamic range	Samples are situated within the range of the efficiency curve
Reverse transcription - qPCR	
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	7900 HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) Software v2.0.1
Statistical justification	4 biological replicates Outliers were eliminated after statistical validation using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05 and 0.01 Log transformation of the data Two-way ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparison using R version 2.13.1
Normalisation	3 reference genes were selected as described in the Methods section

** All procedures were performed according to manufacturer's protocols.*

Supplemental file 4.3. *Transcript levels in the roots of genes encoding oxidative stress hallmark proteins, encoding ROS producing or antioxidative enzymes and of genes involved in ethylene production or responses in Arabidopsis thaliana. Transcript levels were measured using real-time quantitative PCR in root samples of 3-week-old wild-type versus acs2-1acs6-1 mutant plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Data are given as the mean ± s.e. of 4 biological replicates relative to the unexposed genotype set at 1.00 within each time point. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$). Abbreviations: Supplemental file 4.1.*

		24 h		72 h	
		WT	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
Genes encoding ACC Synthases (ACS)					
	Control				
ACS4	**5 µM Cd**				
	10 µM Cd				
	Control	1.00 ± 0.09	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.03
ACS5	**5 µM Cd**	0.01 ± 0.00	0.04 ± 0.01*	0.01 ± 0.01	0.07 ± 0.02*
	10 µM Cd	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
	Control	1.00 ± 0.17	1.00 ± 0.09	1.00 ± 0.21	1.00 ± 0.09
ACS7	**5 µM Cd**	8.15 ± 1.52	4.31 ± 1.40	10.08 ± 3.51	4.37 ± 0.65
	10 µM Cd	9.32 ± 0.93	17.03 ± 1.86	32.08 ± 6.22	14.94 ± 2.55
	Control	1.00 ± 0.32	1.00 ± 0.17	1.00 ± 0.16	1.00 ± 0.27
ACS8	**5 µM Cd**	3.42 ± 0.97	2.44 ± 0.69	5.55 ± 2.92	3.19 ± 0.81
	10 µM Cd	63.36 ± 3.81	57.39 ± 2.41	87.37 ± 27.99	56.65 ± 7.63
	Control	1.00 ± 0.29	1.00 ± 0.22	1.00 ± 0.16	1.00 ± 0.19
ACS11	**5 µM Cd**	1.95 ± 0.23	4.45 ± 0.62*	0.39 ± 0.02	0.75 ± 0.07
	10 µM Cd	1.14 ± 0.12	4.71 ± 0.18*	0.05 ± 0.01	0.13 ± 0.03*
Genes encoding ACC Oxidases (ACO)					
	Control	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.04	1.00 ± 0.01
ACO2	**5 µM Cd**	3.22 ± 0.41	1.00 ± 0.05*	1.56 ± 0.25	1.01 ± 0.03*
	10 µM Cd	3.57 ± 0.06	2.01 ± 0.19*	2.10 ± 0.11	1.63 ± 0.17
	Control	1.00 ± 0.09	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.01
ACO4	**5 µM Cd**	3.89 ± 0.41	1.92 ± 0.28*	4.04 ± 0.43	2.38 ± 0.13*
	10 µM Cd	6.44 ± 0.41	7.74 ± 0.84	7.78 ± 1.17	4.78 ± 0.73*
Ethylene and jasmonate responsive genes					
	Control	1.00 ± 0.06	1.00 ± 0.15	1.00 ± 0.08	1.00 ± 0.10
ERF1	**5 µM Cd**	114.53 ± 26.87	8.25 ± 3.12*	15.21 ± 4.10	3.86 ± 0.50*
	10 µM Cd	232.40 ± 22.86	160.50 ± 15.36	135.94 ± 33.27	44.26 ± 13.21*

		24 h		72 h	
		WT	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
	Control	1.00 ± 0.29	1.00 ± 0.09	1.00 ± 0.15	1.00 ± 0.07
ETR2	**5 µM Cd**	6.71 ± 0.85	2.37 ± 0.47*	2.44 ± 0.50	1.54 ± 0.13
	10 µM Cd	9.94 ± 1.18	6.69 ± 1.20	3.25 ± 0.15	1.88 ± 0.45
	Control	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.10	1.00 ± 0.11
ERF6	**5 µM Cd**	7.69 ± 0.98	2.17 ± 0.23*	2.67 ± 0.44	2.08 ± 0.12
	10 µM Cd	44.68 ± 2.97	43.51 ± 2.92	8.49 ± 0.36	8.23 ± 1.33
	Control	1.00 ± 0.19	1.00 ± 0.07	1.00 ± 0.23	1.00 ± 0.32
VSP2	**5 µM Cd**	0.27 ± 0.13	4.88 ± 3.26*	3.32 ± 0.91	1.84 ± 0.37
	10 µM Cd	0.21 ± 0.12	28.87 ± 16.46*	13.38 ± 4.21	36.75 ± 7.52
Oxidative stress marker genes					
	Control	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.08
AT2G21640	**5 µM Cd**	3.35 ± 0.49	1.15 ± 0.06*	2.12 ± 0.47	2.53 ± 0.19
	10 µM Cd	3.52 ± 0.25	4.69 ± 0.42	8.92 ± 1.55	5.08 ± 0.40*
	Control	1.00 ± 0.19	1.00 ± 0.26	1.00 ± 0.27	1.00 ± 0.24
AT2G43510	**5 µM Cd**	11.73 ± 1.77	4.09 ± 0.19*	175.84 ± 70.31	39.59 ± 12.08*
	10 µM Cd	75.29 ± 2.50	88.92 ± 6.18	482.16 ± 21.94	657.42 ± 113.85
	Control	1.00 ± 0.08	1.00 ± 0.08	1.00 ± 0.10	1.00 ± 0.10
AT1G19020	**5 µM Cd**	5.71 ± 0.18	2.96 ± 0.54*	7.73 ± 1.25	3.99 ± 0.40*
	10 µM Cd	12.30 ± 1.02	23.06 ± 2.31*	22.42 ± 3.78	14.24 ± 1.74
	Control	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.10	1.00 ± 0.06
AT1G05340	**5 µM Cd**	1.52 ± 0.07	1.51 ± 0.21	1.33 ± 0.26	1.12 ± 0.06
	10 µM Cd	1.77 ± 0.09	2.36 ± 0.19	3.24 ± 0.61	3.30 ± 0.23
	Control	1.00 ± 0.11	1.00 ± 0.16	1.00 ± 0.10	1.00 ± 0.07
AT1G57630	**5 µM Cd**	9.73 ± 0.96	3.24 ± 0.78*	9.72 ± 0.35	4.99 ± 0.68*
	10 µM Cd	10.81 ± 1.72	13.36 ± 2.09	46.50 ± 11.12	17.33 ± 3.48*
Antioxidative genes					
	Control	1.00 ± 0.06	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.10
GSH1	**5 µM Cd**	1.39 ± 0.13	0.74 ± 0.07*	0.68 ± 0.06	0.69 ± 0.01
	10 µM Cd	2.60 ± 0.23	2.44 ± 0.13	0.93 ± 0.02	0.94 ± 0.09
	Control	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.06
GSH2	**5 µM Cd**	2.22 ± 0.26	0.91 ± 0.06*	1.02 ± 0.09	0.87 ± 0.03
	10 µM Cd	2.27 ±0.01	1.93 ± 0.12	1.52 ± 0.05	1.52 ± 0.14
	Control	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.03
GR1	**5 µM Cd**	1.54 ± 0.06	0.94 ± 0.06*	1.26 ± 0.05	0.94 ± 0.04
	10 µM Cd	2.77 ± 0.20	2.22 ± 0.14	2.67 ± 0.16	1.81 ± 0.25*
	Control	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.04
GR2	**5 µM Cd**	1.22 ± 0.05	0.89 ± 0.06*	0.87 ± 0.07	0.78 ± 0.04
	10 µM Cd	1.29 ± 0.07	1.09 ± 0.03	0.88 ± 0.04	1.01 ± 0.06
	Control	1.00 ± 0.25	1.00 ± 0.15	1.00 ± 0.33	1.00 ± 0.30
FSD1	**5 µM Cd**	14.63 ± 1.64	13.66 ± 2.88	6.32 ± 0.98	10.42 ± 0.25*
	10 µM Cd	18.67 ± 1.67	22.52 ± 1.47	10.20 ± 1.33	15.63 ± 0.71*

		24 h		72 h	
		WT	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
	Control	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.08
CSD1	**5 µM Cd**	1.34 ± 0.24	0.69 ± 0.10*	0.61 ± 0.16	0.36 ± 0.01
	10 µM Cd	1.76 ± 0.08	1.57 ± 0.17	0.78 ± 0.07	0.48 ± 0.05
	Control	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.05
CSD2	**5 µM Cd**	1.31 ± 0.06	0.70 ± 0.06*	0.63 ± 0.14	0.49 ± 0.03
	10 µM Cd	1.49 ± 0.04	0.92 ± 0.13*	0.54 ± 0.06	0.42 ± 0.02
Pro-oxidative genes					
	Control	1.00 ± 0.02	1.00 ± 0.10	1.00 ± 0.03	1.00 ± 0.10
RBOHC	**5 µM Cd**	0.88 ± 0.09	0.79 ± 0.04	0.62 ± 0.08	0.92 ± 0.04
	10 µM Cd	0.61 ± 0.05	0.56 ± 0.03	0.61 ± 0.05	0.40 ± 0.07*
	Control	1.00 ± 0.11	1.00 ± 0.11	1.00 ± 0.11	1.00 ± 0.06
RBOHD	**5 µM Cd**	3.08 ± 0.06	1.17 ± 0.23*	2.46 ± 0.24	1.77 ± 0.09
	10 µM Cd	4.37 ± 0.47	2.77 ± 0.47	4.38 ± 0.23	4.57 ± 0.63
	Control	1.00 ± 0.04	1.00 ± 0.12	1.00 ± 0.10	1.00 ± 0.11
RBOHF	**5 µM Cd**	2.01 ± 0.26	1.26 ± 0.06	1.85 ± 0.11	0.95 ± 0.03*
	10 µM Cd	2.36 ± 0.11	2.30 ± 0.32	3.53 ± 0.16	1.70 ± 0.21*
	Control	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.10
LOX1	**5 µM Cd**	16.92 ± 5.83	1.69 ± 0.48*	10.84 ± 2.43	2.61 ± 0.27*
	10 µM Cd	65.19 ± 10.83	32.21 ± 3.58	37.21 ± 3.85	19.89 ± 5.48
	Control	1.00 ± 0.07	1.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.07
LOX6	**5 µM Cd**	1.89 ± 0.12	1.07 ± 0.11*	1.13 ± 0.06	1.03 ± 0.02
	10 µM Cd	2.67 ± 0.13	3.17 ± 0.17	2.04 ± 0.15	1.78 ± 0.30
Signal transduction genes					
	Control	1.00 ± 0.11	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.04
MKK9	**5 µM Cd**	10.06 ± 2.26	2.24 ± 0.51*	3.29 ± 0.54	2.30 ± 0.22
	10 µM Cd	19.15 ± 1.12	18.08 ± 1.82	7.36 ± 0.62	9.32 ± 1.62
	Control	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.05
WRKY33	**5 µM Cd**	5.12 ± 0.11	2.31 ± 0.29*	3.16 ± 0.43	2.38 ± 0.09
	10 µM Cd	10.35 ±1.26	10.56 ± 0.06	9.31 ± 1.94	4.88 ± 0.96
	Control	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.05
MPK3	**5 µM Cd**	2.44 ± 0.09	1.53 ± 0.17*	1.91 ± 0.13	1.40 ± 0.04*
	10 µM Cd	3.68 ± 0.12	3.86 ± 0.13	2.90 ± 0.26	2.27 ± 0.12
	Control	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.03
MPK6	**5 µM Cd**	1.56 ± 0.03	1.15 ± 0.03*	0.75 ± 0.04	0.88 ± 0.04
	10 µM Cd	1.61 ± 0.06	1.57 ± 0.05	0.80 ± 0.02	0.72 ± 0.05

Statistics per time point: Tukey's test, except for ACS8: (non-parametric) Wilcoxon rank sum test).

Supplemental file 4.4. *Transcript levels in the leaves of genes encoding oxidative stress hallmark proteins, encoding ROS producing or antioxidative enzymes and of genes involved in ethylene production or responses in Arabidopsis thaliana. Transcript levels were measured using real-time quantitative PCR in root samples of 3-week-old wild-type versus acs2-1acs6-1 mutant plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Data are given as the mean ± s.e. of 4 biological replicates relative to the unexposed genotype set at 1.00 within each time point. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading: p < 0.05; p < 0.01 and p < 0.05; p < 0.01 for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks (p < 0.05). Abbreviations: Supplemental file 4.1.*

		24 h		72 h	
		WT	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
Genes encoding ACC Synthases (ACS)					
	Control	1.00 ± 0.16	1.00 ± 0.16	1.00 ± 0.25	1.00 ± 0.13
ACS4	**5 µM Cd**	0.11 ± 0.02	0.42 ± 0.10*	0.15 ± 0.04	0.64 ± 0.15*
	10 µM Cd	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.02	0.11 ± 0.02
	Control	1.00 ± 0.15	1.00 ± 0.17	1.00 ± 0.27	1.00 ± 0.17
ACS5	**5 µM Cd**	0.06 ± 0.02	0.10 ± 0.04	0.07 ± 0.04	0.34 ± 0.01*
	10 µM Cd	0.05 ± 0.01	0.07 ± 0.02	0.40 ± 0.14	0.13 ± 0.04
	Control	1.00 ± 0.21	1.00 ± 0.42	1.00 ± 0.50	1.00 ± 0.68
ACS7	**5 µM Cd**	28.51 ± 3.31	20.22 ± 2.82	11.65 ± 3.61	3.15 ± 0.76
	10 µM Cd	31.63 ± 5.19	26.39 ± 7.73	21.04 ± 2.82	13.95 ± 3.12
	Control	1.00 ± 0.31	1.00 ± 0.28	1.00 ± 0.16	1.00 ± 0.58
ACS8	**5 µM Cd**	6.21 ± 0.88	6.84 ± 1.70	7.33 ± 0.86	1.88 ± 0.24*
	10 µM Cd	5.02 ± 0.82	12,11 ± 1.63	7.87 ± 0.69	3.05 ± 0.94*
	Control	1.00 ± 0.16	1.00 ± 0.11	1.00 ± 0.14	1.00 ± 0.19
ACS11	**5 µM Cd**	0.38 ± 0.15	3.61 ± 0.69*	1.99 ± 0.14	1.53 ± 0.09
	10 µM Cd	0.19 ± 0.16	0.83 ± 0.46*	0.59 ± 0.17	0.65 ± 0.20
Genes encoding ACC Oxidases (ACO)					
	Control	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.05	1.00 ± 0.05
ACO2	**5 µM Cd**	5.94 ± 1.44	1.41 ± 0.14*	3.68 ± 1.00	1.52 ± 0.14*
	10 µM Cd	5.53 ± 0.46	3.44 ± 0.39	6.88 ± 0.96	4.52 ± 0.48
	Control	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.15	1.00 ± 0.27
ACO4	**5 µM Cd**	9.86 ± 2.02	5.76 ± 0.56*	2.45 ± 0.36	2.40 ± 0.16
	10 µM Cd	6.22 ± 0.32	6.95 ± 0.12	2.81 ± 0.24	1.82 ± 0.15
Ethylene and jasmonate responsive genes					
	Control	1.00 ± 0.33	1.00 ± 0.33	1.00 ± 0.21	1.00 ± 0.65
ERF1	**5 µM Cd**	509.12 ± 70.68	68.83 ± 8.30*	18.27 ± 5.96	2.73 ± 0.44
	10 µM Cd	415 ± 34.15	93.94 ± 0.58*	69.98 ± 16.57	5.32 ± 0.38*

		24 h		72 h	
		WT	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
	Control	1.00 ± 0.27	1.00 ± 0.08	1.00 ± 0.25	1.00 ± 0.15
ETR2	**5 µM Cd**	6.25 ± 1.07	0.90 ± 0.06*	3.00 ± 0.11	2.01 ± 0.31
	10 µM Cd	6.74 ± 0.14	0.82 ± 0.06*	6.52 ± 0.24	2.95 ± 0.45*
	Control	1.00 ± 0.38	1.00 ± 0.04	1.00 ± 0.32	1.00 ± 0.73
PDF1.2	**5 µM Cd**	82.25 ± 16.93	39.31 ± 6.67	512.14 ± 28.41	699.10 ± 96.31
	10 µM Cd	102.02 ± 7.60	132.98 ± 12.24	534.52 ± 20.52	1110.02 ± 177.80
	Control	1.00 ± 0.18	1.00 ± 0.35	1.00 ± 0.30	1.00 ± 0.58
ERF6	**5 µM Cd**	26.70 ± 4.67	18.90 ± 2.14	2.15 ± 0.73	1.14 ± 0.27
	10 µM Cd	30.80 ± 4.27	16.99 ± 2.55	2.54 ± 0.10	1.01 ± 0.23
	Control	1.00 ± 0.19	1.00 ± 0.29	1.00 ± 0.12	1.00 ± 0.84
VSP2	**5 µM Cd**	12.64 ± 2.99	5.20 ± 1.00	91.05 ± 26.67	23.73 ± 2.83*
	10 µM Cd	20.47 ± 2.58	32.24 ± 9.90	183.41 ± 35.63	112.39 ± 10.61
Oxidative stress marker genes					
	Control	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.09	1.00 ± 0.15
AT2G21640	**5 µM Cd**	10.04 ± 2.18	4.04 ± 0.44*	6.48 ± 1.29	6.27 ± 2.08
	10 µM Cd	12.33 ± 0.52	12.45 ± 3.18	7.17 ± 0.39	4.53 ± 0.04
	Control	1.00 ± 0.03	1.00 ± 0.15	1.00 ± 0.12	1.00 ± 0.34
AT2G43510	**5 µM Cd**	47.05 ± 7.01	13.11 ± 1.47*	95.63 ± 30.14	31.68 ± 4.73*
	10 µM Cd	80.87 ± 10.68	39.50 ± 9.86*	238.87 ± 21.79	187.95 ± 26.06
	Control	1.00 ± 0.32	1.00 ± 0.32	1.00 ± 0.11	1.00 ± 0.68
AT1G19020 np	**5 µM Cd**	73.55 ± 13.87	28.05 ± 2.63*	8.21 ± 3.42	2.28 ± 0.55
	10 µM Cd	64.07 ± 1.18	43.06 ± 3.82*	6.60 ± 0.91	1.00 ± 0.12
	Control	1.00 ± 0.28	1.00 ± 0.42	1.00 ± 0.48	1.00 ± 0.63
AT1G05340	**5 µM Cd**	66.77 ± 15.58	20.60 ± 2.10*	9.86 ± 3.43	3.11 ± 0.63
	10 µM Cd	64.37 ± 6.48	37.32 ± 4.53	28.86 ± 3.24	9.89 ± 5.57
	Control	1.00 ± 0.41	1.00 ± 0.49	1.00 ± 0.09	1.00 ± 0.65
AT1G57630 np	**5 µM Cd**	52.52 ± 10.30	18.14 ± 1.71*	2.73 ± 0.15	1.20 ± 0.24
	10 µM Cd	40.86 ± 0.80	24.77 ± 1.16*	4.12 ± 0.37	0.62 ± 0.07
Antioxidative genes					
	Control	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.08	1.00 ± 0.03
GSH1	**5 µM Cd**	1.38 ± 0.28	0.70 ± 0.06*	1.24 ± 0.18	1.01 ± 0.10
	10 µM Cd	1.27 ± 0.11	0.74 ± 0.04*	1.39 ± 0.11	1.24 ± 0.07
	Control	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.07
GSH2	**5 µM Cd**	2.74 ± 0.42	1.16 ± 0.09*	1.63 ± 0.24	1.57 ± 0.19
	10 µM Cd	2.23 ± 0.16	1.45 ± 0.07*	1.35 ± 0.02	1.59 ± 0.15
	Control	1.00 ± 0.06	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.04
GR1	**5 µM Cd**	3.71 ± 0.95	2.01 ± 0.20*	1.61 ± 0.23	1.26 ± 0.10
	10 µM Cd	3.06 ± 0.19	2.56 ± 0.13	1.89 ± 0.09	1.69 ± 0.14
	Control	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.11
GR2 np	**5 µM Cd**	0.56 ± 0.13	0.54 ± 0.05	0.85 ± 0.07	0.93 ± 0.03
	10 µM Cd	0.39 ± 0.02	0.45 ± 0.02	0.85 ± 0.09	0.85 ± 0.01

		24 h		72 h	
		WT	***acs2-1acs6-1***	**WT**	***acs2-1acs6-1***
	Control	1.00 ± 0.51	1.00 ± 0.38	1.00 ± 0.16	1.00 ± 0.16
FSD1	**5 µM Cd**	1.58 ± 0.64	1.50 ± 0.55	0.62 ± 0.12	2.69 ± 0.40*
	10 µM Cd	1.56 ± 0.53	0.88 ± 0.20	0.89 ± 0.12	2.12 ± 0.40*
	Control	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09	1.00 ± 0.50
CSD1	**5 µM Cd**	2.97 ± 0.90	0.83 ± 0.16	1.12 ± 0.46	0.62 ± 0.10
	10 µM Cd	2.05 ± 0.79	2.21 ± 0.52	0.38 ± 0.07	0.29 ± 0.03
	Control	1.00 ± 0.30	1.00 ± 0.15	1.00 ± 0.41	1.00 ± 0.49
CSD2	**5 µM Cd**	0.45 ± 0.03	0.37 ± 0.04	0.26 ± 0.05	0.28 ± 0.04
	10 µM Cd	0.29 ± 0.06	0.46 ± 0.17	0.08 ± 0.01	0.04 ± 0.01
Pro-oxidative genes					
	Control	1.00 ± 0.37	1.00 ± 0.36	1.00 ± 0.82	1.00 ± 0.75
RBOHC	**5 µM Cd**	616.02 ± 84.30	98.36 ± 14.60*	22.43 ± 13.80	17.81 ± 5.88
	10 µM Cd	563.87 ± 39.06	202.88 ± 70.56	11.74 ± 3.11	3.46 ± 1.86
	Control	1.00 ± 0.34	1.00 ± 0.10	1.00 ± 0.15	1.00 ± 0.23
RBOHD	**5 µM Cd**	3.15 ± 0.51	2.07 ± 0.41	2.75 ± 0.26	1.28 ± 0.28*
	10 µM Cd	2.52 ± 0.32	3.06 ± 1.01	2.84 ± 0.51	1.18 ± 0.11
	Control	1.00 ± 0.21	1.00 ± 0.11	1.00 ± 0.09	1.00 ± 0.15
RBOHF	**5 µM Cd**	7.02 ± 2.21	1.74 ± 0.36	3.21 ± 0.36	1.65 ± 0.35
	10 µM Cd	4.28 ± 0.97	4.34 ± 1.62	2.81 ± 0.34	2.04 ± 0.71
	Control	1.00 ± 0.03	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.09
LOX1	**5 µM Cd**	5.46 ± 0.61	1.05 ± 0.14*	3.44 ± 0.95	1.02 ± 0.13*
	10 µM Cd	7.20 ± 0.77	2.20 ± 0.35*	7.89 ± 0.80	4.49 ± 0.58
	Control	1.00 ± 0.08	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.47
LOX2	**5 µM Cd**	6.31 ± 1.13	3.95 ± 0.53	6.35 ± 1.36	5.05 ± 0.71
	10 µM Cd	4.06 ± 0.31	5.10 ± 0.36	6.75 ± 0.95	10.23 ± 1.77
Signal transduction genes					
	Control	1.00 ± 0.10	1.00 ± 0.02	1.00 ± 0.30	1.00 ± 0.27
MKK9	**5 µM Cd**	11.20 ± 2.07	4.37 ± 0.29*	2.17 ± 0.32	1.31 ± 0.17
	10 µM Cd	12.09 ± 0.87	7.05 ± 0.47*	4.02 ± 0.26	2.59 ± 0.32
	Control	1.00 ± 0.17	1.00 ± 0.21	1.00 ± 0.16	1.00 ± 0.46
WRKY33	**5 µM Cd**	26.00 ± 4.58	13.50 ± 1.08*	2.73 ± 0.95	1.29 ± 0.12
	10 µM Cd	20.90 ± 0.78	16.74 ± 1.19	3.33 ± 0.30	0.84 ± 0.14
	Control	1.00 ± 0.15	1.00 ± 0.09	1.00 ± 0.57	1.00 ± 0.46
MPK3	**5 µM Cd**	6.64 ± 1.56	5.44 ± 0.68	0.72 ± 0.12	1.21 ± 0.30
	10 µM Cd	2.41 ± 0.44	4.31 ± 0.35	0.61 ± 0.07	0.91 ± 0.10
	Control	1.00 ± 0.06	1.00 ± 0.14	1.00 ± 0.05	1.00 ± 0.18
MPK6	**5 µM Cd**	2.79 ± 0.62	2.04 ± 0.56	0.82 ± 0.01	1.01 ± 0.08
	10 µM Cd	2.33 ± 0.83	2.29 ± 0.22	0.64 ± 0.04	1.89 ± 0.22*

Statistics per time point: Tukey's test, except for AT1G19020, AT1G57630, GR2: (non-parametric) Wilcoxon rank sum test.

Chapter 5

Ethylene signalling is mediating the early cadmium-induced oxidative challenge in *Arabidopsis thaliana*

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Abstract

Cadmium (Cd) induces the generation of reactive oxygen species (ROS) and stimulates ethylene biosynthesis. The phytohormone ethylene is a regulator of many developmental and physiological plant processes as well as stress responses. Previous research indicated various links between ethylene signalling and oxidative stress. Our results support a correlation between the Cd-induced oxidative challenge and ethylene signalling in *Arabidopsis thaliana* leaves. The effects of 24 or 72 h exposure to 5 µM Cd on plant growth and several oxidative stress-related parameters were compared between wild-type (WT) and ethylene insensitive mutants (*etr1-1*, *ein2-1*, *ein3-1*). Normal Cd-induced responses were affected in *etr1-1* and *ein2-1*, but not in *ein3-1*. Growth of *etr1-1* and *ein2-1* mutants was less inhibited by Cd exposure, and these mutants showed a delayed response in the glutathione (GSH) metabolism, including GSH levels and transcript levels of GSH synthesising and recycling enzymes. Furthermore, the expression of different oxidative stress marker genes was significantly lower in Cd-exposed *ein2-1* mutants, evidencing that ethylene signalling is involved in early responses to Cd stress. A model for the cross-talk between ethylene signalling and oxidative stress is proposed.

Keywords:
Arabidopsis thaliana, cadmium, ethylene signalling, glutathione, oxidative stress

5.1 Introduction

Due to industrial and agricultural activities, elevated levels of toxic metals, such as cadmium (Cd) are widespread in water, soil and atmosphere (Järup and Akesson, 2009). Excessive accumulation of Cd in plant cells leads to enhanced generation of reactive oxygen species (ROS), inducing an oxidative challenge (Cuypers et al., 2010). Processes including the activation of pro-oxidative enzymes (e.g. NADPH oxidases) or disturbances in the mitochondrial electron transfer chain (ETC) are responsible for this increased ROS production (Cuypers et al., 2011; Keunen et al., 2013). Depending on the severity of the stress, different damaging and protective signalling pathways are activated by ROS. To maintain a tightly controlled redox balance, plants developed an extensive antioxidative network consisting of enzymes and metabolites (Cuypers et al., 2000; Sharma and Dietz, 2009). Chelation of Cd by the antioxidative metabolite glutathione (GSH) and its polymer phytochelatins, followed by sequestration in the vacuoles is essential in Cd detoxification and results from the high affinity of Cd for the thiol group in these compounds (Cobbett and Goldsbrough, 2002). Furthermore, GSH is also capable of reducing H_2O_2, a common ROS, oxidising itself to glutathione disulphide (GSSG) as a part of the enzymatic antioxidative ascorbate-glutahione (AsA-GSH) cycle (Jozefczak et al., 2014; Rauser, 2001).

The plant hormone ethylene is considered a 'stress hormone' involved in multiple molecular and physiological plant processes, regulating various growth and cellular defence responses (Argueso et al., 2007; Lin et al., 2009). The biosynthesis of ethylene increases after short-term Cd exposure in *Arabidopsis thaliana*. Using double knock-out (KO) *acs2-1acs6-1* mutant plants, we previously showed that the Cd-induced ethylene production mainly relies on the expression of these two 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) isozymes that accomplish the rate-limiting step of ethylene biosynthesis (Schellingen et al., 2014). In *A. thaliana*, ethylene is perceived by a family of five negatively regulating receptors, including ETHYLENE RESISTANT1 (ETR1). Ethylene binding to these receptors deactivates their functioning in maintaining the inhibitory action of the Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Dugardeyn and Van Der Straeten, 2008; Lin et al., 2009), thus allowing activation of the downstream ethylene response through the ethylene signal transducer ETHYLENE INSENSITIVE2 (EIN2), a transmembrane

protein with low similarities to NRAMP metal transporters (Vandenbussche et al., 2012). The C-terminal end of EIN2 is cleaved off and moves to the nucleus, stabilising the transcription factors ETHYLENE INSENSITIVE3/EIN3 LIKE1 (EIN3/EIL1), which in turn activate the expression of transcription factors as ETHYLENE RESPONSE FACTOR1 (*ERF1*) and other ethylene responsive genes (Ji and Guo, 2013).

Previous research revealed various links between ethylene signalling and the oxidative challenge imposed by different stress conditions. A reduced ROS production was observed in flagellin- and mercury- (Hg) treated ethylene insensitive *A. thaliana* mutants (Mersmann et al., 2010; Montero-Palmero et al., 2014). In contrast, salinity still induced oxidative stress in the ethylene insensitive *ein2-5* mutant plants (Lin et al., 2012). Stress-mediated ethylene signalling is also known to alter glutathione biosynthesis. In contrast to wild-type *A. thaliana* plants, ozone exposure did not increase GSH production in ethylene insensitive mutants (Yoshida et al., 2009). Moreover, Cao et al. (2009) stated that ethylene signalling mediates increased lead (Pb) tolerance in *A. thaliana* seedlings by a mechanism partially on GSH. To date, no clear link between Cd-induced oxidative challenge and ethylene signaling has been established. We hypothesise that ethylene signalling during Cd exposure affects the early cellular redox balance at the transcript as well as the metabolic level. To investigate this, we exposed mutants with an impaired ethylene signal transduction pathway, *etr1-1*, *ein2-1* and *ein3-1*, short-term (24 and 72 h) to 5 µM Cd. From these and previous results we build a model, exhibiting the link between the Cd-induced oxidative challenge and ethylene biosynthesis and signalling.

5.2 Methods

5.2.1 Plant material, culture, treatment and sampling

Arabidopsis thaliana (Columbia ecotype) wild-type (WT), and mutants *acs2-1acs6-1* (Tsuchisaka et al., 2009), *etr1-1* (Schaller and Bleecker, 1995), *ein2-1* (Alonso, 1999), *ein2-5* (Alonso, 1999), *ein3-1* (Chao et al., 1997), *oxi1* (Rentel et al., 2004) were used. All mutants were checked for homozygosity of the mutation by PCR.

Seeds were surface sterilised during 1 minute in 0.1% NaClO and afterwards thoroughly washed in sterilised water. Further, the seedlings were cultivated using a modified Hoagland nutrient solution on hydroponics according to Smeets et al. (2008), using purified calibrated sand. The nutrient solution was refreshed twice a week. Established growth conditions were 12 h photoperiod with day/night temperatures of respectively 22/18 °C and 65 % relative humidity. A combination of blue, red and far-red led modules (Philips Green-Power LED modules, the Netherlands) was used to simulate the photosynthetic active radiation (PAR) spectrum of sunlight with a photosynthetic photon flux density of 170 µmol m^{-1} s^{-1} at the leaf level (Keunen et al., 2011).

Three weeks old plants grown on hydroponics were exposed to 5 µM $CdSO_4$ at the root level (except for control plants). This sublethal concentration is commonly found in the pore water of contaminated soils (Krznaric et al., 2009) and was also applied in earlier hydroponic growth experiments (Keunen et al., 2011). After 24 or 72 h of exposure, whole root and shoot systems were separated, sampled and snap frozen in liquid nitrogen prior to storage at -70 °C except for quantification of Cd contents. Biological replicates for each measured parameter (number of replicates displayed in table and figure captions) were sampled from various pots of the same conditions to avoid within pot correlation (Smeets et al., 2008).

5.2.2 Quantification of Cd contents

Leaves of hydroponically grown plants were harvested and rinsed with distilled water. Samples were oven-dried at 80 °C and digested in HNO_3 (70-71 %) in a heat block (Cuypers et al., 2002). Cadmium concentrations in the extracts were determined by inductively coupled plasma-atomic emission spectrometry (ICP-

AES, Perkin-Elmer, 1100B, USA). As references, blanks (HNO_3 only) and certified standard samples (NIST Spinach (1570a)) were analysed.

5.2.3 Gene expression analysis

RNA was extracted using the RNAqueous® Total RNA Isolation Kit (Life Technologies, Belgium), according to the manufacturers instructions, from frozen leaf tissues of hydroponically grown plants disrupted under frozen conditions in 2mL microcentrifuge tubes using two stainless steel beads and the Retsch Mixer Mill MM 400 (Retsch, Belgium). RNA concentration and purity were evaluated spectrophotometrically on the NanoDrop ND-1000 (ThermoScientific, USA). DNase treatment with the TURBO DNA-free™ Kit (Life Technologies) was performed to eliminate possible genomic DNA contamination. One µg of the treated RNA was converted to single stranded cDNA using the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Bio Inc., the Netherlands) according to the manufacturers instructions. The cDNA was diluted 10-fold in 1/10 diluted TE buffer (1 mM Tris–HCl, 0.1 mM Na2-EDTA, pH 8.0; Sigma–Aldrich, Belgium) and stored at -20 °C. Quantitative real-time PCR was performed in an optical 96-well plate with the 7900HT Fast Real-Time PCR System (Life Technologies) using SYBR Green chemistry. Gene-specific forward and reverse primers were designed via the Primer Express software (v2.0, Life Technologies). Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C) followed by the generation of a dissociation curve to verify amplification specificity. Reactions contained 2 µL diluted cDNA template (or RNase-free H_2O for the 'no template controls'), 5 µL 2x Fast SYBR® Green Master Mix (Life Technologies), forward and reverse primers (300 nM each, unless otherwise mentioned in Supplemental file 5.1) in a total volume of 10 µL. The specificity of the used primer pairs was checked *in silico* using Blast (http://www.arabidopsis.org/Blast/index.jsp) and after qPCR by verifying single peaks on the dissociation curve. In addition, primer efficiency (E) was evaluated on a standard curve generated using a twofold dilution series of a mixed sample over at least five dilution points and verified to be higher than 80 % (E = 10^(-1/slope)). In supplemental file 5.1, all gene annotations, primer sequences and primer efficiencies are shown. Gene expression levels were calculated according to the $2^{-\Delta Cq}$ method relative to the sample with the highest expression

(minimum Cq). The data obtained were normalised using the geometric average of the $2^{-\Delta Cq}$ values of three stable reference genes selected out of a set of 10 (Remans et al., 2008) by geNorm (v3.5) and Normfinder (v0.953) algorithms (Andersen et al., 2004; Vandesompele et al., 2002). The most stable reference genes (*AT2G28390*, *AT4G34270*, *AT5G25760*) were used to determine sample-specific normalisation factors (Supplemental file 5.1). Supplemental file 5.2 shows the RT-qPCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

5.2.4 Glutathione content

The oxidised and reduced forms of GSH were extracted and spectrophotometrically measured according to the plate reader method previously described by Queval and Noctor (2007) and modified by Jozefczak et al. (2014). Frozen leaf samples (100 mg) were ground in liquid nitrogen using a cooled mortar and pestle and further homogenised by adding 200 mM HCl (800 µl per 120 mg fresh weight). After centrifugation (10 min, 16 000g, 4 °C), the pH of the samples was adjusted to 4.5. Unless otherwise mentioned, the samples were kept at 4 °C during the entire procedure. The spectrophotometric measurement of GSH and GSSG is monitored at 412 nm during 5 min and is based on the reduction of 5,5-dithiobis(2-nitro-benzoic acid) (DTNB, 600 µM) by the action of glutathione reductase (GR, 1U mL^{-1}) in the presence of NADPH (500 µM). Total glutathione (reduced and oxidised) concentrations were calculated relative to a standard curve ranging from 0 to 500 pmol GSH. The oxidised GSSG concentration was measured by incubating the samples with 2-vinyl-pyridine (2-VP, 1% v/v) during 30 min at room temperature to precipitate all free reduced GSH present in the sample. Prior to the measurement, 2-VP was precipitated by centrifuging the samples twice (10 min, 16000g, 4 °C). For quantification purposes, samples for a GSSG standard curve ranging from 0 to 100 pmol were incubated with 2-VP and measured in duplicate concurrently with the samples. By subtracting the concentration of oxidised GSSG from the total GSH concentration, the amount of reduced GSH was calculated (Queval and Noctor, 2007).

5.2.5 Statistical analysis

Outliers were determined using the extreme studentised deviate analysis (GraphPad Software, USA) at significance level 0.05. The datasets were analysed via the linear model procedure in R (R Development Core Team, 2012). Both normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) were checked; transformations were applied when necessary to approximate normality. For gene expression data, normalised relative quantities were log transformed prior to further statistical analysis. Normally distributed data were analysed using the one- or two-way ANOVA procedure. Tukey–Kramer adjustment for multiple comparisons was applied to obtain corrected p-values. The statistical analyses of non-normally distributed data were based on the non-parametric Kruskal–Wallis test followed by the post-hoc pairwise Wilcoxon rank sum test. Figure and Table captions indicate the experiment specific statistical analysis.

5.3 Results & Discussion

Previously, Cd exposure was shown to transiently increase ethylene production in WT *A. thaliana* plants, but not in double-KO *acs2-1acs6-1* mutants (Schellingen et al., 2014; Chapter 3). Furthermore, after exposure to moderate (5 µM) concentrations of Cd, a reduction of the growth inhibiting effect and a delay in the early induction of oxidative stress were observed in these mutants compared to the WT, especially in the leaves, whereas exposure to severe (10 µM) concentrations overwhelmed both the WT and mutant plants (Chapter 4). To further unravel the significance of the Cd-induced increase of ethylene production, we used ethylene signalling mutants and investigated the effects of moderate Cd concentrations on growth and on metabolic and molecular parameters in the leaves. Wild-type *A. thaliana* plants and three different ethylene insensitive mutants were exposed to 5 µM Cd during 24 or 72 h. These mutants, *etr1-1*, *ein2-1* and *ein3-1* were selected because they cover three distinct consecutive steps in the ethylene signal transduction pathway: perception, transduction and execution of the ethylene signal (De Paepe and Van Der Straeten, 2005).

5.3.1 Cadmium-induced effects on plant growth are mediated by ethylene signalling

Unexposed *etr1-1* plants had a significantly lower fresh weight and diameter compared to the other genotypes. The rosette diameter and growth rate of *ein2-1* mutants was also reduced in comparison to the WT and *ein3-1* mutant plants (Fig. 5.1 A & B). No significant decrease in leaf fresh weight was observed in the WT or any of the ethylene insensitive mutant plants after 24 h exposure to 5 µM Cd (Fig. 5.1 A), while for the rosette diameter we did measure a significant decrease in the Cd-exposed *ein3-1* mutant plants (Fig. 5.1 B).

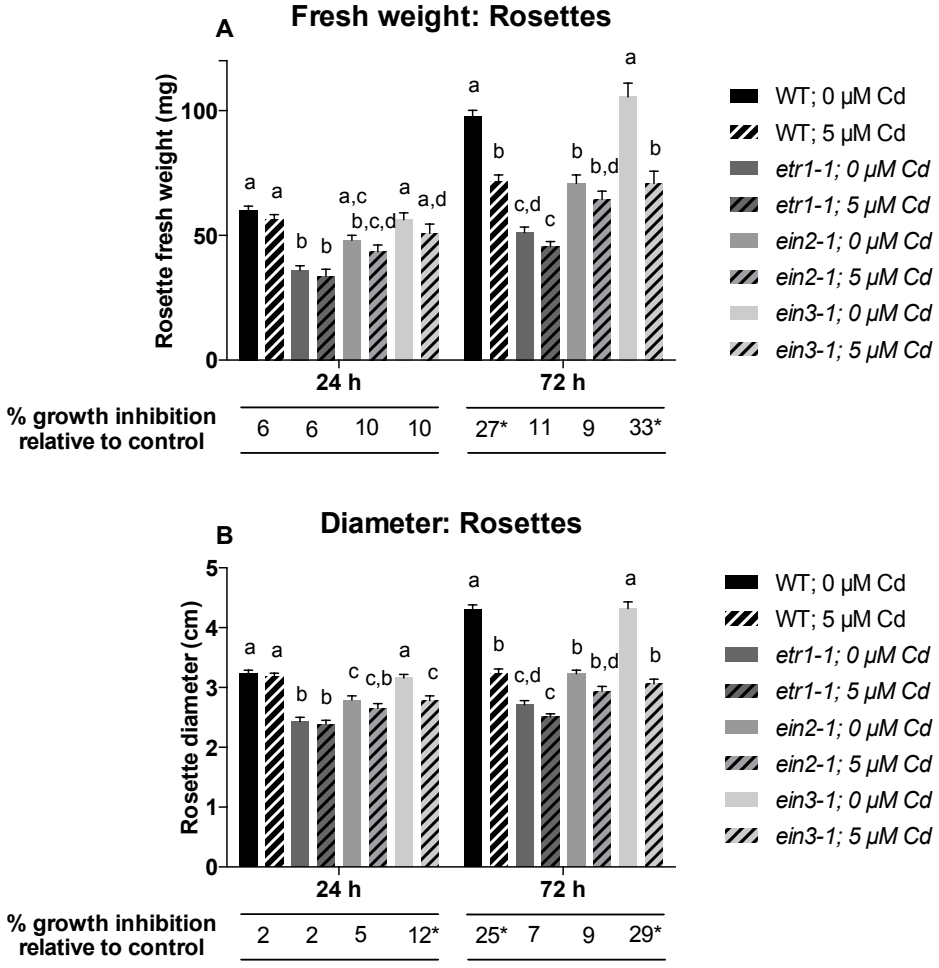


Figure 5.1 Fresh weight, diameter & growth inhibition. *A comparison of the* ***(A)*** *fresh weight (mg) and* ***(B)*** *diameter, with their corresponding growth inhibition (%) of rosettes of 3-week-old wild-type or etr1-1, ein2-1, ein3-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to 5 μM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Fresh weight & diameter: Data shows mean ± s.e. of at least 12 biological replicates. The letters a-d represent groups with a significantly different FW (Tukey's test: p < 0.05). Statistics was performed separately within each exposure time. Growth inhibition: Data shows mean ± s.e. of at least 12 biological replicates relative to the control within each exposure time and genotype. Significance levels: * p < 0.05 (Tukey's test). Statistics was performed separately within each exposure time and genotype.*

The Cd content of the mutant plants did not significantly differ from the WT after 24 h (Table 5.1). Prolonged exposure (72 h) to Cd significantly further increased the Cd contents in the leaves of all genotypes. Although the Cd content was in the same range for all genotypes, it was significantly lower in *ein2-1* and *ein3-1* mutants than in WT plants (Table 5.1). At this exposure time (72 h), Cd exposure significantly decreased the rosette fresh weight and diameter of WT and *ein3-1* mutant plants, whereas the genotypes with a significantly lower fresh weight and diameter under control conditions (*etr1-1* and *ein2-1*) were not affected after Cd exposure (Fig. 5.1 A & B).

Table 5.1 Cd content. *A comparison of the Cd concentrations (mg kg^{-1} DW^{-1}) in leaves of 3-week-old wild-type, etr1-1, ein2-1, ein3-1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to 5 μM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data represent mean ± s.e. of four biological replicates. The letters a-d represent groups with a significantly different Cd content after treatment (Tukey's test: p < 0.05). nd: levels below detection limit.*

Cd content	Leaves			
24 h	wildtype	*etr1-1*	*ein2-1*	*ein3-1*
0 μM $CdSO_4$	nd	nd	nd	nd
5 μM $CdSO_4$	752 ± 61ad	720 ± 35a	589 ± 59a	571 ± 43a
72 h	wildtype	*etr1-1*	*ein2-1*	*ein3-1*
0 μM $CdSO_4$	nd	nd	nd	nd
5 μM $CdSO_4$	1360 ± 39b	1147 ± 25bc	1046 ± 32cd	1092 ± 40c

The similar response observed in *ein3-1* mutant and WT plants can be explained by the redundancy within the EIN3 family of transcription factors, which harbours five EIL (EIN3-Like) transcription factors. Chao et al. (1997) stated that EIL1 and EIL2 are able to complement *ein3-1* in *A. thaliana* (Binder et al., 2007). For ETR1 however, although also four other ethylene receptors exist, it has been shown that *etr1-1* is a strong dominant gain of function mutation causing ethylene insensitivity (Gamble et al., 2002; Mersmann et al., 2010; Yoo et al., 2009). EIN2 is known to be an essential and unique positive regulator of ethylene signalling. The *ein2-1* mutation therefore results in a loss-of-function ethylene insensitive genotype (Ji and Guo, 2013; Qiao et al., 2012). The effect of toxic metal stresses on growth of ethylene signalling mutants has been extensively studied using different experimental setups, however, without leading to an exact conclusion. Cao et al. (2009) reported that 7 or 14 days of exposure to 0.75 mM lead nitrate ($Pb(NO_3)_2$) had a significantly stronger inhibiting effect on root and shoot fresh weight of the *ein2-1* mutant *A. thaliana* plants compared to the WT. In contrast, Zhang et al. (2014) concluded that the shoot fresh weight of 20-day-old hydroponically grown *ein2-1 A. thaliana* plants exposed to 1 mM aluminium (Al) for 14 days was significantly higher compared to the WT. Exposure to 15 mM lithium chloride (LiCl) during 10 days had a significantly smaller inhibitory effect on the growth of *etr1-1* and *ein3-3 A. thaliana* plants compared to the WT (Bueso et al., 2007). Likewise, compared to the WT, a diminished root growth inhibition in 5-day-old *ein2-5* mutant *A. thaliana* seedlings exposed to 0.3 µM Hg during 2 or 3 days was observed by Montero-Palmero et al. (2014).

In our short-term experimental setup, we can conclude that ethylene signalling is necessary for normal plant growth, and that increased ethylene production (as measured in Chapter 3; Schellingen et al., 2014) and signalling negatively affects plant growth after exposure to moderate Cd concentrations (Fig. 5.1).

5.3.2 Ethylene signalling is involved in the upregulation of the glutathione metabolism in Cd-exposed plants

The metabolite GSH is an important determinant in Cd-induced responses as it links Cd chelation on the one hand and Cd-induced oxidative challenge on the other hand (Jozefczak et al., 2014; Semane et al., 2007). Interestingly, the existence of a cross-talk between ethylene and the GSH metabolism has been mentioned in multiple studies (reviewed by Iqbal et al., 2013). Therefore, we investigated the Cd-induced effect on GSH metabolism in relation to ethylene signal transduction by comparing different ethylene-insensitive mutants with WT plants. We focussed on the GSH content (Table 5.2) as well as on the expression of genes encoding enzymes that are involved in (1) GSH biosynthesis (*GSH1* and *GSH2*), (2) recycling of GSSG to its reduced form GSH (*GR1* and *GR2*) and (3) polymerization of GSH to PC (*PC1* and *PC2*) (Fig. 5.2 A, Supplemental files 5.3 & 5.4).

In WT plants exposed to 5 µM Cd during 24 h, the expression of the gene coding for the initial enzyme in GSH biosynthesis, *i.e.* glutamate-cysteine ligase (*GSH1*), did not change after Cd exposure, in contrast to the transcript levels of glutathione synthetase (*GSH2*), which covers the second and last step of GSH biosynthesis (Fig. 5.2 A). The expression of glutathione reductase 1 (*GR1*) also increased, whereas transcript levels of *GR2* remained unaffected (Fig. 5.2 A). It was previously shown that Cd exposure increases the H_2O_2 content in *A. thaliana* seedlings (Cuypers et al., 2011) and that GR1 plays a crucial role in leaf responses to H_2O_2 (Mhamdi et al., 2010), explaining our observation. The enhanced expression of *GSH2* and *GR1* after 24 h exposure to Cd did not significantly increase the concentration of reduced or total GSH, but it may have allowed WT plants to maintain their GSH content within physiological ranges (Table 5.2). In agreement with this, Zhu et al. (1999) reported that the expression of *GSH2* is important in alleviating the depletion of GSH under Cd stress. This depletion was clearly demonstrated as a fast response in roots of Cd-exposed *A. thaliana* plants but was not observed in leaves (Jozefczak et al., 2014). In addition to its antioxidant properties, GSH is also used for the biosynthesis of phytochelatins, Cd-chelating peptides. In WT plants the expression of phytochelatin synthase 1 (*PCS1*), is increased after 24 h of Cd exposure (Fig. 5.2 A). In line with this, Jozefczak et al. (2014) also observed an

increased *PCS1* expression in the leaves of WT *A. thaliana* plants exposed to 5 or 10 µM Cd during 24 h, which resulted in an increased phytochelatin production. Earlier it was also reported that *PCS2* is less abundant and unrelated to metal detoxification (Cobbett and Goldsbrough, 2002; Jozefczak et al., 2014; Kühnlenz et al., 2014; Remans et al., 2012). Consistent with these results, the expression of *PCS2* was not affected by Cd exposure (Fig. 5.2 C). Whereas the expression of genes involved in the glutathione metabolism was less or not significantly affected after 72 h exposure to Cd (Fig. 5.2 A, Supplemental file 5.3), both the concentrations of total as well as reduced GSH increased (Table 5.2). This might result from further enhanced activities of the earlier (after 24 h Cd exposure) synthesised GSH2 and GR1 enzymes, which is supported by results of Yoshida et al. (2009). They observed a rapid increase in the *GSH2* transcript levels, followed by a decrease while GSH2 activity kept increasing after prolonged exposure. A similar effect for GR1 after Cd exposure was also observed by Jozefczak, M. (personal communication). In addition, Jozefczak et al. (2014) also observed a Cd-induced increase of the GSH content in leaves of *A. thaliana* after 72 h exposure to 5 or 10 µM Cd.

Table 5.2 Glutathione content. *A comparison of the glutathione content (reduced, oxidised and total; nmol GSH equivalents g^{-1} FW) in leaves of 3-week-old wild-type,* ***(A)*** *etr1-1,* ***(B)*** *ein2-1,* ***(C)*** *ein3-1 Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Data are given as mean ± s.e. of at least 4 biological replicates. Significant Cd-induced changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for induction and inhibition respectively (Tukey's test). Statistics was performed separately for reduced, oxidised and total glutathione within each time point and genotype.*

		24 h		72 h	
A		**WT**	***etr1-1***	**WT**	***etr1-1***
GSH+GSSG	**Control**	260.77 ± 28.60	285.82 ± 9.64	235.05 ± 10.04	262.24 ± 4.60
	5 µM Cd	255.34 ± 13.96	176.58 ± 8.98	336.26 ± 41.29	267.17 ± 7.91
GSH	**Control**	247.80 ± 29.23	269.77 ± 8.60	220.06 ± 7.93	245.26 ± 6.02
	5 µM Cd	251.19 ± 14.43	176.13 ± 8.97	331.05 ± 40.43	262.39 ± 7.77
GSSG	**Control**	12.97 ± 1.56	19.36 ± 3.42	15.00 ± 2.21	16.98 ± 1.63
	5 µM Cd	4.15 ± 1.46	0.45 ± 0.07	5.21 ± 0.99	4.78 ± 0.15
B		**WT**	***ein2-1***	**WT**	***ein2-1***
GSH+GSSG	**Control**	230.37 ± 11.14	253.48 ± 19.73	201.63 ± 31.84	296.79 ± 30.29
	5 µM Cd	224.33 ± 35.41	170.49 ± 15.65	349.16 ± 50.69	250.43 ± 1.11
GSH	**Control**	221.29 ± 13.97	242.95 ± 19.80	180.78 ± 24.52	270.10 ± 37.23
	5 µM Cd	219.59 ± 31.30	171.11 ± 15.00	341.75 ± 53.00	246.59 ± 0.64
GSSG	**Control**	9.58 ± 5.32	10.54 ± 0.73	20.85 ± 9.09	26.68 ± 6.96
	5 µM Cd	4.74 ± 4.78	0.63 ± 0.65	7.42 ± 2.36	3.85 ± 0.66
C		**WT**	***ein3-1***	**WT**	***ein3-1***
GSH+GSSG	**Control**	256.76 ± 6.89	292.83 ± 20.56	217.89 ± 26.54	226.49 ± 18.44
	5 µM Cd	251.41 ± 3.36	246.96 ± 16.31	318.59 ± 14.30	251.75 ± 6.79
GSH	**Control**	245.06 ± 8.10	269.92 ± 20.37	205.63 ± 24.67	208.13 ± 18.41
	5 µM Cd	248.41 ± 4.00	244.74 ± 16.46	311.26 ± 14.24	245.89 ± 6.87
GSSG	**Control**	13.67 ± 2.15	22.91 ± 6.74	12.26 ± 2.01	18.36 ± 3.66
	5 µM Cd	2.87 ± 0.49	2.22 ± 0.58	7.33 ± 0.38	5.86 ± 1.84

Similar to WT plants, no significant difference in GSH content was observed after 24 h Cd exposure of *ein3-1* mutant plants. However, in contrast to the WT, prolonged exposure of these *ein3-1* mutants to Cd did not lead to significant increases of the contents of total and reduced GSH (Table 5.2 C). In the *etr1-1* and *ein2-1* mutants, the response of total and reduced GSH contents to Cd exposure differed from WT seedlings after both 24 and 72 h exposure. Cadmium-induced decreases of the concentrations of total and reduced GSH compared to the control were observed in both *etr1-1* and *ein2-1* mutant plants after 24 h exposure. After 72 h Cd exposure, this effect disappeared in both mutants, but in contrast with the WT, Cd exposure did again not significantly increase the total and reduced GSH contents (Table 5.2 A & B). These results indicate a delayed GSH response in the ethylene insensitive mutants, especially in *etr1-1* and *ein2-1* mutant plants.

The increased expressions of *GSH2* and *GR1* determined in the WT plants after 24 h Cd exposure were also observed in the *ein3-1* mutant plants (Supplemental file 5.3). The *etr1-1* and the *ein2-1* mutant plants did not display this Cd-induced increase in expression, possibly explaining the differences in GSH content between the former (WT, *ein3-1*) and the latter (*etr1-1*, *ein2-1*) after 24 h Cd exposure (Fig. 5.2 A; Table 5.2; Supplemental file 5.3 & 5.4). As mentioned before, after 72 h of Cd exposure the GSH content in WT plants possibly increased due to the early enhanced expression of the genes involved in the GSH metabolism resulting in prolonged higher activities. Hampering the gene expression of GSH-related enzymes in the ethylene insensitive mutant plants might also affect the activity of these enzymes, delaying the effect on GSH content (Fig. 5.2 A; Table 5.2). In accordance with this, Yoshida et al. (2009) also observed a decreased GSH content in *ein2-1* mutant *A. thaliana* plants following ozone-induced suppression of *GSH2* and *GR1* expression. They also found a suppressed and delayed GSH2 activity in this mutant. Moreover, Cao et al. (2009) observed significantly lower transcript levels of *GSH1* in two-week-old *ein2-1 A. thaliana* continuously exposed to 0.5 mM $Pb(NO_3)_2$, resulting in decreased GSH concentrations compared to the WT. To the best of our knowledge, stress-induced effects on the expression of the genes involved in the GSH metabolism or on the GSH content have never been reported for the *etr1-1* or *ein3-1* mutants.

In conclusion, the delayed response in the ethylene insensitive mutant plants indicates an early involvement of ethylene signalling in the Cd-induced increase of GSH in *A. thaliana*.

5.3.3 The expression of oxidative stress hallmark genes is altered in Cd-exposed ethylene insensitive mutants

Ethylene is known to be a positive regulator of ROS production, potentially increasing oxidative stress (Mersmann et al., 2010; Overmyer et al., 2003). Since GSH is important in counteracting oxidative stress and the GSH content was lower in the ethylene insensitive mutants, we further investigated the relation between ethylene signalling and the Cd-induced oxidative challenge in these mutants. To elucidate this link, the expressions of a set of 5 marker genes, referred to as hallmark genes for general oxidative stress described by Gadjev et al. (2006), were determined in the WT as well as the different ethylene insensitive mutants.

Exposure to Cd increased the expression of all oxidative stress hallmark genes in WT and *ein3-1* mutant plants (Fig. 5.2 B). In the *etr1-1* mutants, fewer upregulated transcript levels were observed, while in *ein2-1* mutant plants the expressions of all hallmark genes were significantly lower compared to WT plants (Fig. 5.2 B). Therefore, we conclude that ethylene signal transduction, especially EIN2, is involved in the early Cd-induced oxidative challenge. EIN2 is known as a unique transducer of ethylene and stress responses (Alonso, 1999). In line with the former, by determining H_2O_2 accumulation Zhang et al. (2014) concluded that, in comparison to the WT, a lower level of oxidative stress occured in 20-day-old *ein2-1* mutant *A. thaliana* plants exposed to 1 mM Al for 14 days. Cao et al. (2006) also observed an enhanced oxidative stress tolerance as well as an alleviated oxidative damage in two-week-old *ein2-1* mutant *A. thaliana* continuously exposed to paraquat (PQ). Furthermore, Montero-Palmero et al. (2014) reported a decrease in oxidative stress by measuring extracellular H_2O_2 in the roots of 10-day-old *ein2-5* mutant *A. thaliana* plants exposed to 0.2 µM $HgCl_2$ during 6 h. On the contrary, Lin et al. (2012) observed an exaggerated salt-induced oxidative stress (100 mM NaCl) in 6-day-old *ein2-5 A. thaliana* plants. To find out whether our results observed in the *ein2-1* mutant were allele-specific, the effect of Cd on the expression of the 5 oxidative stress

marker genes and the GSH metabolism was also investigated in *ein2-5* mutant plants. The obtained results confirmed those observed in the *ein2-1* mutants (Supplemental files 5.5 & 5.6).

5.3.4 A model explaining the early cross-talk between ethylene biosynthesis, signal transduction and oxidative stress induced by Cd

Cadmium exposure is known to increase ethylene production in WT *A. thaliana* plants (Schellingen et al., 2014; Chapter 3). Further, exposure to Cd also causes oxidative stress in *A. thaliana* (Cuypers et al., 2011). The purpose of this study was to unravel the relation between ethylene production and oxidative stress under moderate Cd stress conditions. In figure 5.2 C, a working model concerning this cross-talk after 24 h Cd exposure is proposed.

The Cd-induced increase in ethylene production was shown to be based on increases of ACS2 and ACS6, two ACC synthesising isozymes, typically involved in stress responses (Schellingen et al., 2014; Chapter 3). ACC oxidase (ACO) converts ACC to ethylene, that is triggering the ethylene signal transduction. Our results reveal a delay in the response of the GSH metabolism after Cd exposure in the ethylene insensitive mutants compared to the WT as well as a diminished oxidative stress profile especially in the *ein2-1* mutant, indicating an early involvement of ethylene in stress-sensing (Table 5.2, Fig. 5.2 A2). To further elucidate the effects of Cd-induced ethylene signalling on oxidative stress, the expression of the respiratory burst oxidase homologue C (*RBOHC)* gene, a Cd-induced NADPH oxidase with an important role in ROS production, was determined in the WT and the ethylene insensitive mutants (Fig. 5.2 A2, Supplemental files 5.3 & 5.4). Previously, this isoform was shown to be strongly induced by Cd in the leaves of WT *A. thaliana* plants (Cuypers et al., 2011; Remans et al., 2010). Furthermore, previous studies concluded that ethylene serves as an activator of NADPH oxidases (Chae and Lee, 2001; Jakubowicz et al., 2010). In WT and *ein3-1* mutant plants, the expression of *RBOHC* was upregulated after 24 h exposure to Cd (Fig. 5.2 A2). In *etr1-1* mutants, there was no upregulation of the expression of *RBOHC*, while in *ein2-1* the transcript levels were significantly lower compared to the WT plants (Fig. 5.2 A2). In agreement to our findings, Mersmann et al. (2010) observed a diminished ROS generation by flagellin FLS22 in *etr1* and *ein2* mutant *A. thaliana* seedlings,

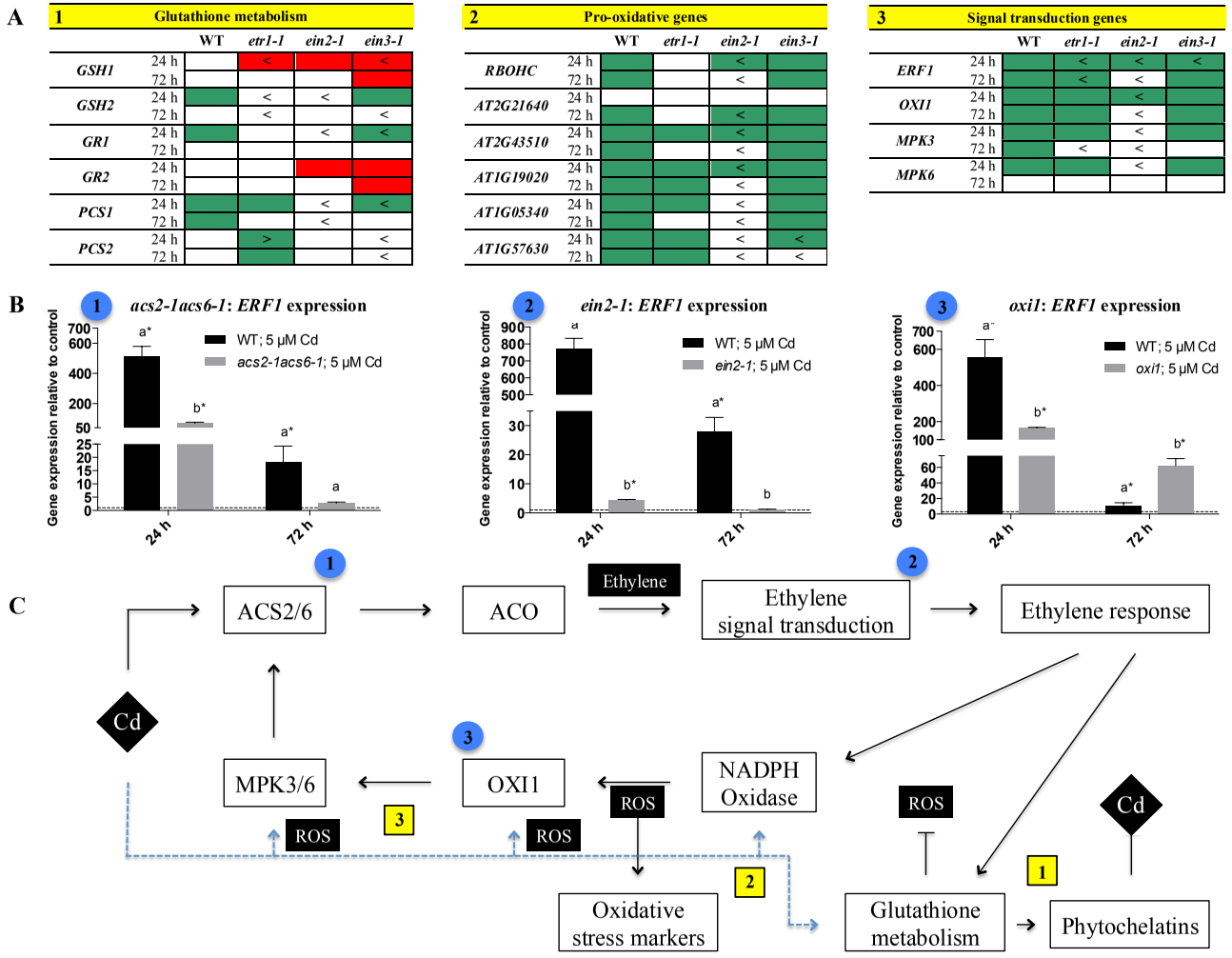
which was related to an inhibited activation of NADPH oxidases due to impaired ethylene signalling. Moreover, the increase in NADPH oxidase activity was also abolished in Hg-treated alfalfa roots after treatment with 1-methylcyclopropene, an ethylene receptor inhibitor (Montero-Palmero et al., 2014). In sweet potato, the use of the NADPH oxidase inhibitor diphenyleneiodonium also decreased the ROS production induced by the ethylene releasing compound ethephon (Chen et al., 2013). Taken together, these results strongly suggest the involvement of ethylene signalling in ROS production through NADPH oxidases. Increased ROS production potentially affects the GSH metabolism.

An important signalling protein known to play a central role in metal-induced ROS sensing is the oxidative signal-inducible kinase1 (OXI1) (Smeets et al., 2013). In its turn, it activates the mitogen-activated protein kinases (MPKs) MPK3 and MPK6 (Rentel et al., 2004). Opdenakker et al. (2012) observed that Cd exposure led to oxidative stress mediated increases in *MPK3/6* transcript levels. In line with these results, we found increased expressions of *OXI1*, *MPK3* and *MPK6* after 24 h Cd exposure in WT plants (Fig. 5.2 A3). In the *etr1-1* and *ein3-1* mutants the expression of *OXI1*, *MPK3* and *MPK6* also increased, although to a minor extent compared to the WT plants (Fig. 5.2 A3, Supplemental files 5.3 & 5.4). In the *ein2-1* mutant plants, however, the expression of these 3 genes was mainly unaltered and significantly lower than in WT plants (Fig. 5.2 A3), indicating an impaired signal transduction in these mutants. Liu et al. (2010) also found an increasing activity of MPK3 and MPK6 in response to Cd mediated by the accumulation of ROS in *A. thaliana*. Type 1 ACS proteins, like ACS2 and ACS6, are phosphorylation targets of MPK3 and MPK6. This posttranslational modification increases the half-life of the ACS enzymes (Lin et al., 2009; Skottke et al., 2011). In addition, the transcriptional activity of *ACS2* and *ACS6* is also induced by MPK3 and MPK6, of which the activity is possibly induced by CTR1 (Li et al., 2012; Vandenbussche et al., 2012). Since, as already mentioned, the Cd-induced ethylene production relies on these two isoforms (*ACS2* and *ACS6*), this mechanism hereby closes the loop in the proposed model and is capable to further increase the ethylene production, feeding into an autocatalytic loop (Fig. 5.2 C) (Schellingen et al., 2014; Vandenbussche et al., 2012; Chapter 3).

To further support this working model, the expression of *ERF1*, an ethylene responsive gene (Vandenbussche et al., 2012), was determined in KO-mutants of subsequent different steps involved in ethylene biosynthesis (*acs2-1acs6-1*), ethylene signal transduction (*ein2-1*) and oxidative stress signal transduction (*oxi1*) (Fig. 5.2 B). The expression of *ERF1* is significantly increased in WT plants after 24 h Cd exposure, corroborating the early involvement of ethylene signalling in Cd-induced stress responses (Fig. 5.2 B). Although the transcript levels of *ERF1* were also increased after 24 h Cd exposure in the *acs2-1acs6-1* double KO-mutants, they were significantly lower compared to the WT (Fig. 5.2 B1), again demonstrating the importance of both ACC synthesising isoforms in the Cd-induced ethylene production. The expression of *ERF1* was also significantly lower in the *ein2-1* mutant plants (Fig. 5.2 B2) and the *oxi1* KO-mutant plants (Fig. 5.2 B3) compared to the WT after 24 h Cd exposure, further supporting our model.

In conclusion, our data support that ethylene signalling is involved in the regulation of the early Cd-induced oxidative challenge in *A. thaliana* leaves through the control of GSH content and oxidative stress profile.

Figure 5.2. *Schematic overview of the hypothesis and supporting data, explaining the early link between ethylene biosynthesis, signal transduction and oxidative stress, in 3-week-old Arabidopsis thaliana leaves exposed to moderate (5 µM) Cd concentrations (see results & discussion for further details).* ***(A)*** *Transcript levels in the leaves of (1) genes encoding enzymes involved in the glutathione metabolism, (2) pro-oxidative genes or oxidative stress marker genes and (3) signal transduction genes in Arabidopsis thaliana. Transcript levels were measured in 3-week-old wild-type, etr1-1, ein2-1 and ein3-1 knockout plants exposed to 5 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, significant Cd-induced expression changes within each genotype relative to its own unexposed control are indicated with colour shading;* $p < 0.01$ *and* $p < 0.01$ *for induction and inhibition respectively, while differences between the mutant genotypes and the WT are indicated with > or < ($p < 0.05$) for induction and inhibition respectively (Tukey's Test).* ***(B)*** *A comparison of the relative expression of the ERF1 gene in leaves of 3-week-old wild-type, (1) acs2-1acs6-1, (2) ein2-1, (3) oxi1 mutant Arabidopsis thaliana plants exposed for 24 or 72 h to 5 µM $CdSO_4$ or grown under control conditions in a hydroponic culture system. Per time point, data show mean ± s.e. of at least 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks (Tukey's test: * $p < 0.05$). The letters a-b represent groups with a significantly different gene expression between both genotypes (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time and genotype.* ***(C)*** *Proposed model. Abbreviations: ACO (ACC Oxidase), ACS (ACC Synthase), Cd (cadmium), EIN (Ethylene insensitive), ERF (Ethylene response factor), GSH1 (Glutamate-cysteine ligase), GSH2 (Glutathione synthetase), GR (Glutathione reductase), MPK (Mitogen activated protein kinase), OXI (Oxidative-signal inducible), PCS (Phytochelatin synthase), RBOH (Respiratory burst oxidase homologue), ROS (Reactive oxygen species). Raw data are shown in Supplemental files 5.3 & 5.4: representing the mean ± s.e. of 4 biological replicates relative to the unexposed genotype.*


A
1 Glutathione metabolism
WT etr1-1 ein2-1 ein3-1
GSH1 24 h < <
72 h
GSH2 24 h < <
72 h <
GR1 24 h < <
72 h
GR2 24 h
72 h
PCS1 24 h < <
72 h <
PCS2 24 h > <
72 h <
2 Pro-oxidative genes
WT etr1-1 ein2-1 ein3-1
RBOHC 24 h <
72 h <
AT2G21640 24 h
72 h <
AT2G43510 24 h <
72 h <
AT1G19020 24 h <
72 h <
AT1G05340 24 h <
72 h <
AT1G57630 24 h < <
72 h < <
3 Signal transduction genes
WT etr1-1 ein2-1 ein3-1
ERF1 24 h < < <
72 h < <
OXI1 24 h <
72 h <
MPK3 24 h <
72 h < <
MPK6 24 h <
72 h
B
1 acs2-1acs6-1: ERF1 expression
Gene expression relative to control
WT; 5 µM Cd
acs2-1acs6-1; 5 µM Cd
24 h
72 h
2 ein2-1: ERF1 expression
Gene expression relative to control
WT; 5 µM Cd
ein2-1; 5 µM Cd
24 h
72 h
3 oxi1: ERF1 expression
Gene expression relative to control
WT; 5 µM Cd
oxi1; 5 µM Cd
24 h
72 h
C
1
ACS2/6
ACO
Ethylene
2
Ethylene signal transduction
Ethylene response
Cd
3
MPK3/6
OXI1
ROS
NADPH Oxidase
ROS
ROS
ROS
3
2
Oxidative stress markers
ROS
Cd
1
Glutathione metabolism
Phytochelatins

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SUPPLEMENTAL FILES

Supplemental file 5.1. *Forward (FW) and reverse (REV) primers used to determine gene expression levels via quantitative real-time PCR. E-E-jn, exon-exon junction; LOX, lipoxygenase; RBOH, respiratory burst oxidase homologue; OXI, Oxidative signal-inducible; mitogen-activated protein kinase kinase; MPK, mitogen-activated protein kinase; SAND, SAND family; TIP41-like, tonoplast intrinsic protein 41-like; UBC, ubiquitin-conjugating enzyme; UPOX, upregulated by oxidative stress; TIR, Toll-Interleukin-1; GSH1, glutamate-cysteine ligase; GSH2, glutathione synthetase; GR, glutathione reductase; PCS, Phytochelatin Synthase; CSD, Cu/Zn superoxide dismutase; FSD, Fe superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; ETR, ethylene receptor; ERS, ethylene response sensor; EIN, ethylene insensitive; CTR, constitutive triple response; EIL, ethylene insensitive like; EBF, EIN3 binding F-BOX protein; ETP, EIN2 targeting protein; RTE, reversion to ethylene sensitivity; ERF, ethylene response factor; PDF, plant defensin; VSP, vegetative storage protein. * primer concentrations were increased to 900 nM. ** primer concentrations were increased to 600 nM.*

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Genes encoding ROS producing enzymes					
AT1G55020	*LOX1*	FW: TTGGCTAAGGCTTTTGTCGG REV: GTGGCAATCACAAACGGTTC	Exon 6 and 7	101	99.13%
AT3G45140	*LOX2*	FW: TTTGCTCGCCAGACACTTG REV: GGGATCACCATAAACGGCC	Exon 3 and 4	102	86.65%
AT5G51060	*RBOHC*	FW: TCACCAGAGACTGGCACAATAAA REV: GATGCTCGACCTGAATGCTC	Exon 6 and 7	101	92.09%
AT5G47910	*RBOHD*	FW: AACTCTCCGCTGATTCCAACG REV: TGGTCAGCGAAGTCTTTAGATTCCT	Exon 1	91	99.24%
Genes encoding signal transduction genes					
AT3G25250	*OXI1*	FW: CGATTATTGTCCGGGACAGA REV: CTAATACAAGCTCCGCCGC	Exon 1 and 2	104	104.40%
AT3G45640	*MPK3*	FW: GACGTTTGACCCCAACAGAA REV: TGGCTTTTGACAGATTGGCTC	Exon 5 and 6	103	100.37%
AT2G43790	*MPK6*	FW: TAAGTTCCCGACAGTGCATCC REV: GATGGGCCAATGCGTCTAA	Exon 5 and 6	101	107.68%
Reference genes					
AT2G28390	*SAND family*	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13 and 14	61	97.41%
AT4G34270	*TIP41-like*	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn and Exon 2	61	90.60%
AT5G25760	*UBC*	FW: CTGCGACTCAGGGAATCTTCTAA REV: TTGTGCCATTGAATTGAACCC	E3-E4-jn and Exon 4	61	102.61%

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Genes encoding markers for ROS-induced gene expression					
AT2G21640	*UPOX*	FW: GACTTGTTTCAAAAACACCATGGAC REV: CACTTCCTTAGCCTCAATTTGCTTC	Exon 1 and 2	91	93.77%
AT2G43510	*Defensin-like*	FW: ATGGCAAAGGCTATCGTTTCC REV: CGTTACCTTGCGCTTCTATCTCC	Exon 1 and 2	91	98.42%
AT1G19020	*Unknown*	FW: GAAAATGGGACAAGGGTTAGACAAA REV: CCCAACGAAAACCAATAGCAGA	Exon 1	92	99.30%
AT1G05340	*Unknown*	FW: TCGGTAGCTCAGGGTAAAGTGG REV: CCAGGGCACAACAGCAACA	Exon 2 and 3	91	101.62%
AT1G57630	*TIR-class*	FW: ACTCAAACAGGCGATCAAAGGA REV: CACCAATTCGTCAAGACAACACC	Exon 1	91	94.56%
Genes encoding antioxidative enzymes					
AT4G23100	*GSH1*	FW: CCCTGGTGAACTGCCTTCA REV: CATCAGCACCTCTCATCTCCA	Exon 5 and 6	101	98.60%
AT5G27380	*GSH2*	FW: GGACTCGTCGTTGGTGACAA REV: TCTGGGAATGCAGTTGGTAGC	Exon 11 and 12	101	92.60%
AT3G24170	*GR1*	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15 and 16	101	95.69%
AT3G54660	*GR2*	FW: TAGGGTTGGAGAATGTTGGCG REV: GCCCAGATGGATGGAACAGAT	Exon 5 and 6	91	99.82%
AT5G44070	*PCS1*	FW: TGGTGTTGAATGCTCTTTCTATCG REV: GGTTCGCAGCAATCCAACAT	Exon 2	91	95.08%
AT1G03980	*PCS2***	FW: CCATGGTTAGCCACCCGACT REV: TCTTCTTTATTCTCCTGGCATCGT	Exon 1	94	109.84%
AT1G08830	*CSD1*	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5 and E6-E7-jn	102	94.22%
AT2G28190	*CSD2*	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6 and E7-E8-jn	101	96.21%
AT4G25100	*FSD1*	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4 and E6-E7-jn	101	92.76%
AT1G20630	*CAT1*	FW: AAGTGCTTCATCGGGAAGGA REV: CTTCAACAAAACGCTTCACGA	E5-E6-jn and exon 7	103	94.02%
AT4G35090	*CAT2*	FW: AACTCCTCCATGACCGTTGGA REV: TCCGTTCCCTGTCGAAATTG	Exon 2 and 3	76	96.32%
AT1G20620	*CAT3*	FW: TCTCCAACAACATCTCTTCCCTCA REV: GTGAAATTAGCAACCTTCTCGATCA	Exon 2 and 3	91	82.04%
AT1G07890	*APX1*	FW: TGCCACAAGGATAGGTCTGG REV: CCTTCCTTCTCTCCGCTCAA	Exon 5 and 6	101	94.43%

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Ethylene signal transduction genes					
AT1G66340	*ETR1*	FW: TGGGTACTTGTTCAGTTTGGTGC REV: CGGTTCTCGAATGCGTAGTGA	Exon 1	91	88.52%
AT3G23150	*ETR2**	FW: TTCGAACCGGGCAGTTACAC REV: AATGGCGGTAAGGCAATCG	Exon 2	91	87.50%
AT2G40940	*ERS1*	FW: TAGAAAACGTGGCGGATCAGG REV: TGCTCCATAAGCTGGTCACGA	Exon 1 and 2	92	93.35%
AT1G04310	*ERS2*	FW: AGTCTCAACGCTTGCCAAAACAT REV: CAACTGAGACGCTTTTCACCAAAC	Exon 1 and 2	93	94.90%
AT3G04580	*EIN4*	FW: ATGGGATTAAGGAAGGCTTGAGC REV: CCATGCGATTTAGGTGATATCCA	Exon 1 and 2	91	108.08%
AT5G03730	*CTR1*	FW: CGATTGAAGGCCAGCACGT REV: TAGACGGCTCATCTCGCAGG	Exon 12 and 13	91	101.74%
AT5G03280	*EIN2*	FW: CGTGTCCTTGACTTGTCTCTCATG REV: CCGGATCAATCACTCCCTGTAG	Exon 3 and 4	94	90.71%
AT3G20770	*EIN3*	FW: GGCCTCAACTTGGTTTGCCT REV: GTCAAAACGCCGACTTTCCA	Exon 1	91	96.83%
AT2G27050	*EIL1*	FW: GTCCAACACCGCATACGCTT REV: CTCTGCGGTGGATCACAATGT	Exon 1	91	92.99%
AT2G25490	*EBF1*	FW: CTTGGAAAATTGTCGATTCGAGG REV: ACCCGAGAGAAGGGCAGCTA	Exon 2	91	103.54%
AT5G25350	*EBF2*	FW: TGGAACTTGCCTGCTGTTAGTG REV: CAGGACACCGTGAAAGGTCAA	Exon 2	91	93.73%
AT1G54490	*EIN5*	FW: AGCAGCGTTCTCGACGTTTC REV: TTTGGCCCTCCATCTCAAAAT	Exon 3 and 4	96	85.08%
AT3G18980	*ETP1*	FW: GGTATGCAACCACGTAGGCAA REV: GGCTTCGATCCACCTGGTTT	Exon 1	91	103.99%
AT3G18910	*ETP2***	FW: GAGCAGCTTCCAAATGACTTGG REV: TCCATGCTTTGCAAGTCGATC	Exon 1	91	109.09%
AT2G26070	*RTE1*	FW: TTAGTTGTGCCTTCAGCGACG REV: GGATCTGATAACACGAGCCGC	Exon 1	91	84.77%
AT3G15210	*ERF4*	FW: TCTCCAACTCGATAGAACCAAGTGT REV: TGTTCCAAAGTCGGTGTGTTTG	E1-E2-jn and Exon 2	91	90.19%
Ethylene and jasmonate responsive genes					
AT3G23240	*ERF1***	FW: TCCTCGGCGATTCTCAATTTT REV: CAACCGGAGAACAACCATCCT	Exon 1	91	98.10%
AT5G44420	*PDF1.2*	FW: TTTGCTGCTTTCGACGCAC REV: GCATGCATTACTGTTTCCGCA	E1-E2-jn and Exon 2	99	94.45%
AT5G24770	*VSP2*	FW: GGCGTGACCTACTGGAAGCA REV: CGAGACTCTTCCTCACCTTTGACTT	Exon 2 and 3	91	95.09%

Supplemental file 5.2. *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). * All procedures were performed according to manufacturer's protocols.*

Sample/Template	
Source	Leaves (entire rosette) of *Arabidopsis thaliana* plants cultivated in hydroponics
Method of preservation	Liquid nitrogen
Storage time	3 weeks at - 70 °C freezer
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 file 5.1
Amplicon details	Exon location and amplicon size: Supplemental file 5.1
Primer sequences	Supplementary file 5.1
In silico	Primers were blasted using the BLAST tool at http://arabidopsis.org/
Empirical	A primer concentration of 300 nM was used unless stated otherwise Annealing temperature: 60 °C
Priming conditions	Combination of oligodT-primers and random hexamers
PCR efficiency	Dilution series (slope, y-intercept and r^2; Supplemental file 5.1)
Linear dynamic range	Samples are situated within the range of the efficiency curve
Reverse transcription - qPCR	
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	7900 HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) Software v2.0.1
Statistical justification	4 biological replicates Outliers were eliminated after statistical validation using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05 and 0.01 Log transformation of the data Two-way ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparison using R version 2.13.1
Normalisation	3 reference genes were selected as described in the Methods section

Supplemental file 5.3. *Transcript levels in the roots of genes encoding components of the ethylene signal transduction pathway, ethylene and jasmonate responsive genes, antioxidative genes, pro-oxidative genes, signal transduction genes and oxidative stress marker genes in Arabidopsis thaliana. Transcript levels were measured using quantitative real-time PCR in leaf samples of 3-week-old wild-type or ein2-1 and ein3-1 knockout plants exposed to 5 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± s.e. of 4 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$) (Tukey's Test per time point). Abbreviations: Supplemental file 5.1.*

		24 h			72 h		
		WT	***ein2-1***	***ein3-1***	**WT**	***ein2-1***	***ein3-1***
Pro-oxidative genes							
LOX1	**Control**	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.16	1.00 ± 0.05	1.00 ± 0.04
	5 µM Cd	1.13 ± 0.08	1.17 ± 0.11	1.24 ± 0.06	1.30 ± 0.17	0.85 ± 0.04	1.16 ± 0.04
LOX2	**Control**	1.00 ± 0.08	1.00 ± 0.15	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.09	1.00 ± 0.02
	5 µM Cd	3.75 ± 0.12	2.67 ± 0.24	5.23 ± 1.03	2.87 ± 0.57	1.47 ± 0.32	3.57 ± 0.82
RBOHC	**Control**	1.00 ± 0.36	1.00 ± 0.19	1.00 ± 0.30	1.00 ± 0.15	1.00 ± 0.37	1.00 ± 0.33
	5 µM Cd	215.14 ± 60.76	20.84 ± 5.88*	79.94 ± 23.39	64.12 ± 24.97	1.99 ± 0.54*	28.02 ± 11.24
RBOHD	**Control**	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.08	1.00 ± 0.03	1.00 ± 0.10
	5 µM Cd	3.33 ± 0.18	1.77 ± 0.13*	2.77 ± 0.19	3.19 ± 0.34	1.47 ± 0.10*	2.92 ± 0.44
Oxidative stress marker genes							
AT2G21640	**Control**	1.00 ± 0.02	1.00 ± 0.12	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.10	1.00 ± 0.03
	5 µM Cd	5.13 ± 1.13	5.72 ± 1.17	5.97 ± 0.57	6.69 ± 0.84	2.27 ± 0.26*	5.22 ± 1.03
AT2G43510	**Control**	1.00 ± 0.09	1.00 ± 0.22	1.00 ± 0.17	1.00 ± 0.13	1.00 ± 0.18	1.00 ± 0.33
	5 µM Cd	28.88 ± 4.13	6.05 ± 0.15*	21.55 ± 1.43	22.87 ± 4,24	2.25 ± 0.45*	24.09 ± 10.02
AT1G19020	**Control**	1.00 ± 0.14	1.00 ± 0.19	1.00 ± 0.21	1.00 ± 0.37	1.00 ± 0.13	1.00 ± 0.02
	5 µM Cd	71.12 ± 12.41	4.23 ± 0.63*	34.15 ± 4.78	16.40 ± 3.96	1.73 ± 0.22*	14.53 ± 3.43
AT1G05340	**Control**	1.00 ± 0.11	1.00 ± 0.31	1.00 ± 0.35	1.00 ± 0.25	1.00 ± 0.08	1.00 ± 0.23
	5 µM Cd	41.79 ± 6.46	2.83 ± 0.67*	20.50 ± 2.02	24.65 ± 6.61	1.96 ± 0.31*	16.51 ± 6.20
AT1G57630	**Control**	1.00 ± 0.10	1.00 ± 0.24	1.00 ± 0.60	1.00 ± 0.22	1.00 ± 0.42	1.00 ± 0.77
	5 µM Cd	278.47 ± 53.69	2.52 ± 0.51*	17.74 ± 3.11*	32.81 ± 7.98	0.53 ± 0.09*	3.74 ± 0.77*

		24 h			72 h		
		WT	***ein2-1***	***ein3-1***	**WT**	***ein2-1***	***ein3-1***
Antioxidative genes							
FSD1	**Control**	1.00 ± 0.30	1.00 ± 0.52	1.00 ± 0.44	1.00 ± 0.44	1.00 ± 0.76	1.00 ± 0.22
	5 µM Cd	0.78 ± 0.07	2.56 ± 0.31	0.78 ± 0.08	4.33 ± 1.36	2.19 ± 0.50	0.63 ± 0.18
CSD1	**Control**	1.00 ± 0.17	1.00 ± 0.05	1.00 ± 0.20	1.00 ± 0.05	1.00 ± 0.21	1.00 ± 0.22
	5 µM Cd	1.53 ± 0.15	0.89 ± 0.10	1.43 ± 0.21	0.41 ± 0.08	0.21 ± 0.03	0.57 ± 0.06
CSD2	**Control**	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.18	1.00 ± 0.10	1.00 ± 0.22	1.00 ± 0.27
	5 µM Cd	0.72 ± 0.17	0.40 ± 0.14	0.64 ± 0.26	0.12 ± 0.02	0.09 ± 0.01	0.15 ± 0.02
GSH1	**Control**	1.00 ± 0.02	1.00 ± 0.07	1.00 ± 0.03	1.00 ± 0.10	1.00 ± 0.05	1.00 ± 0.02
	5 µM Cd	0.85 ± 0.03	0.72 ± 0.04	0.57 ± 0.07*	0.79 ± 0.09	0.81 ± 0.06	0.62 ± 0.04
GSH2	**Control**	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.09	1.00 ± 0.04	1.00 ± 0.09
	5 µM Cd	1.81 ± 0.06	1.20 ± 0.07*	1.39 ± 0.05	1.52 ± 0.14	1.08 ± 0.08	0.96 ± 0.10*
PCS1	**Control**	1.00 ± 0.05	1.00 ± 0.12	1.00 ± 0.21	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.07
	5 µM Cd	3.79 ± 0.21	1.61 ± 0.08*	2.44 ± 0.10*	1.50 ± 0.04	0.93 ± 0.03*	1.49 ± 0.10
PCS2	**Control**	1.00 ± 0.06	1.00 ± 0.11	1.00 ± 0.12	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.16
	5 µM Cd	1.57 ± 0.16	1.16 ± 0.07	0.94 ± 0.14*	1.78 ± 0.05	1.33 ± 0.24	0.79 ± 0.10*
GR1	**Control**	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.06
	5 µM Cd	2.24 ± 0.21	1.12 ± 0.09*	1.47 ± 0.07*	1.00 ± 0.08	0.76 ± 0.09	0.85 ± 0.06
GR2	**Control**	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.03
	5 µM Cd	0.64 ± 0.02	0.63 ± 0.06	0.48 ± 0.05	0.63 ± 0.01	0.80 ± 0.08	0.56 ± 0.03
CAT1	**Control**	1.00 ± 0.04	1.00 ± 0.09	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.12	1.00 ± 0.09
	5 µM Cd	1.76 ± 0.09	1.55 ± 0.11	1.24 ± 0.15*	1.22 ± 0.24	0.74 ± 0.06	0.94 ± 0.19
CAT2	**Control**	1.00 ± 0.03	1.00 ± 0.09	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.05
	5 µM Cd	0.37 ± 0.03	0.65 ± 0.07*	0.40 ± 0.07	0.55 ± 0.02	0.75 ± 0.05	0.53 ± 0.04
CAT3	**Control**	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.12	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.13
	5 µM Cd	1.01 ± 0.09	1.15 ± 0.08	0.93 ± 0.11	2.58 ± 0.58	1.32 ± 0.22	1.56 ± 0.39
APX1	**Control**	1.00 ± 0.05	1.00 ± 0.09	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.07	1.00 ± 0.05
	5 µM Cd	1.30 ± 0.13	1.17 ± 0.09	0.89 ± 0.09	0.90 ± 0.15	0.66 ± 0.12	0.62 ± 0.11
Signal Transduction genes							
OXI1	**Control**	1.00 ± 0.10	1.00 ± 0.18	1.00 ± 0.36	1.00 ± 0.18	1.00 ± 0.30	1.00 ± 0.21
	5 µM Cd	108.31 ± 24.13	10.70 ± 2.25*	66.43 ± 16.80	43.07 ± 13.79	2.74 ± 0.52*	16.11 ± 6.03
MPK3	**Control**	1.00 ± 0.08	1.00 ± 0.13	1.00 ± 0.27	1.00 ± 0.12	1.00 ± 0.15	1.00 ± 0.30
	5 µM Cd	5.22 ± 0.20	1.64 ± 0.16*	3.34 ± 0.21	2.98 ± 0.22	0.93 ± 0.05*	1.75 ± 0.23
MPK6	**Control**	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.09
	5 µM Cd	1.82 ± 0.04	1.23 ± 0.11*	1.68 ± 0.10	1.12 ± 0.08	0.87 ± 0.04	0.91 ± 0.05

		24 h			72 h		
		WT	***ein2-1***	***ein3-1***	**WT**	***ein2-1***	***ein3-1***
Ethylene signal transduction genes							
ETR1	**Control**	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.08	1.00 ± 0.05	1.00 ± 0.06
	5 µM Cd	1.25 ± 0.07	1.09 ± 0.02	1.07 ± 0.06	1.35 ± 0.06	0.90 ± 0.02*	1.10 ± 0.03
ETR2	**Control**	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.16	1.00 ± 0.17
	5 µM Cd	3.32 ± 0.26	0.73 ± 0.05*	0.84 ± 0.05*	3.06 ± 0.45	1.35 ± 0.11	1.37 ± 0.42*
ERS1	**Control**	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.06
	5 µM Cd	2.74 ± 0.08	1.46 ± 0.06*	1.71 ± 0.03*	1.89 ± 0.10	1.07 ± 0.04*	1.30 ± 0.09*
ERS2	**Control**	1.00 ± 0.10	1.00 ± 0.05	1.00 ± 0.01	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.02
	5 µM Cd	2.57 ± 0.18	0.62 ± 0.06*	0.93 ± 0.09*	1.46 ± 0.15	0.96 ± 0.09	0.95 ± 0.13*
EIN4	**Control**	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.09
	5 µM Cd	1.95 ± 0.03	1.29 ± 0.04*	1.47 ± 0.05*	1.28 ± 0.05	0.99 ± 0.01*	1.33 ± 0.08
CTR1	**Control**	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.07
	5 µM Cd	2.37 ± 0.11	1.30 ± 0.06*	1.41 ± 0.04*	1.62 ± 0.11	1.06 ± 0.04*	1.18 ± 0.07*
EIN2	**Control**	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.02
	5 µM Cd	0.91 ± 0.05	0.88 ± 0.02*	0.68 ± 0.05*	1.02 ± 0.07	0.89 ± 0.02	0.96 ± 0.03
EIN3	**Control**	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.07
	5 µM Cd	1.39 ± 0.04	1.32 ± 0.04	1.40 ± 0.07	1.35 ± 0.14	1.01 ± 0.06	1.09 ± 0.10
EIL1	**Control**	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.12
	5 µM Cd	0.62 ± 0.07	0.78 ± 0.07	0.55 ± 0.06	0.89 ± 0.06	0.98 ± 0.04	0.76 ± 0.06
EBF1	**Control**	1.00 ± 0.02	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.02
	5 µM Cd	1.21 ± 0.08	1.12 ± 0.04	0.75 ± 0.03*	0.98 ± 0.07	0.83 ± 0.02	0.81 ± 0.03
EBF2	**Control**	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.09
	5 µM Cd	1.91 ± 0.08	1.19 ± 0.04*	0.74 ± 0.03*	1.54 ± 0.11	0.92 ± 0.01*	1.01 ± 0.11*
EIN5	**Control**	1.00 ± 0.11	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.01
	5 µM Cd	1.18 ± 0.04	0.96 ± 0.07	0.97 ± 0.10	1.21 ± 0.09	0.97 ± 0.02	0.99 ± 0.01
ETP1	**Control**	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.05
	5 µM Cd	0.51 ± 0.05	0.65 ± 0.02	0.50 ± 0.06	0.53 ± 0.09	0.64 ± 0.02	0.48 ± 0.05
ETP2	**Control**	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.09	1.00 ± 0.05	1.00 ± 0.02
	5 µM Cd	0.55 ± 0.05	0.65 ± 0.04	0.46 ± 0.04	0.90 ± 0.10	0.94 ± 0.01	0.88 ± 0.07
ERF4	**Control**	1.00 ± 0.02	1.00 ± 0.12	1.00 ± 0.14	1.00 ± 0.09	1.00 ± 0.09	1.00 ± 0.06
	5 µM Cd	3.91 ± 0.26	1.61 ± 0.06*	1.25 ± 0.07*	2.32 ± 0.26	1.11 ± 0.11*	1.49 ± 0.18*
RTE1	**Control**	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.08	1.00 ± 0.02	1.00 ± 0.05
	5 µM Cd	0.83 ± 0.04	0.78 ± 0.04	0.59 ± 0.04*	0.97 ± 0.06	0.98 ± 0.07	0.77 ± 0.05

		24 h			72 h		
		WT	***ein2-1***	***ein3-1***	**WT**	***ein2-1***	***ein3-1***
Ethylene and jasmonate responsive genes							
ERF1	**Control**	1.00 ± 0.24	1.00 ± 0.21	1.00 ± 0.46	1.00 ± 0.30	1.00 ± 0.21	1.00 ± 0.64
	5 µM Cd	768.14 ± 65.15	4.40 ± 0.24*	30.22 ± 4.14*	27.97 ± 4.84	1.15 ± 0.16*	9.90 ± 3.19
VSP2	**Control**	1.00 ± 0.20	1.00 ± 0.28	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.33	1.00 ± 0.35
	5 µM Cd	2.71 ± 0.32	2.46 ± 0.67	5.20 ± 1.41	8.23 ± 2.86	4.07 ± 2.16	2.41 ± 0.62
PDF1.2	**Control**	1.00 ± 0.33	1.00 ± 0.43	1.00 ± 0.71	1.00 ± 0.06	1.00 ± 0.23	1.00 ± 0.80
	5 µM Cd	87.78 ± 16.24	10.51 ± 3.80	98.73 ± 3.36	626.37 ± 64.45	32.04 ± 7.89*	235.77 ± 54.93

Supplemental file 5.4. *Transcript levels in the leaves of genes encoding ethylene responsive proteins, oxidative stress markers, glutathione synthesis enzymes, pro-oxidative enzymes and signal transduction enzymes in Arabidopsis thaliana. Transcript levels were measured using quantitative real-time PCR in leaf samples of 3-week-old wild-type or etr1-1 knockout plants exposed to 5 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± s.e. of 4 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$). Abbreviations: Supplemental file 5.1.*

		24 h		72 h	
		WT	***etr1-1***	**WT**	***etr1-1***
Ethylene and jasmonate responsive genes					
ERF1	**Control**	1.00 ± 0.33	1.00 ± 0.21	1.00 ± 0.23	1.00 ± 0.27
	5 µM Cd	308.42 ± 120.40	27.60 ± 1.78*	19.18 ± 4.08	3.73 ± 0.79*
Oxidative stress marker genes					
AT2G21640	**Control**	1.00 ± 0.02	1.00 ± 0.07	1.00 ± 0.04	1.00 ± 0.11
	5 µM Cd	5.86 ± 2.41	3.59 ± 0.73	5.12 ± 1.04	2.88 ± 0.52
AT2G43510	**Control**	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.10	1.00 ± 0.19
	5 µM Cd	29.51 ± 14.76	15.54 ± 3.77	34.05 ± 17.63	8.01 ± 1.93
AT1G19020	**Control**	1.00 ± 0.07	1.00 ± 0.16	1.00 ± 0.18	1.00 ± 0.10
	5 µM Cd	95.07 ± 30.46	23.59 ± 2.37	7.58 ± 0.48	9.12 ± 2.33
AT1G05340 np	**Control**	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.12	1.00 ± 0.17
	5 µM Cd	58.09 ± 24.67	31.58 ± 3.77	6.92 ± 1.30	8.21 ± 0.53
AT1G57630	**Control**	1.00 ± 0.16	1.00 ± 0.25	1.00 ± 0.20	1.00 ± 0.29
	5 µM Cd	145.08 ± 54.90	41.74 ± 6.53	7.43 ± 0.91	5.50 ± 1.11
Glutathione synthesis genes					
GSH1	**Control**	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.06
	5 µM Cd	1.05 ± 0.05	0.66 ± 0.04*	1.04 ± 0.19	0.69 ± 0.12
GSH2	**Control**	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.06
	5 µM Cd	2.52 ± 0.61	0.93 ± 0.06*	1.56 ± 0.25	0.91 ± 0.07*
GR1	**Control**	1.00 ± 0.01	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.06
	5 µM Cd	2.40 ± 0.48	1.81 ± 0.15	1.34 ± 0.15	0.94 ± 0.09
GR2	**Control**	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.10
	5 µM Cd	0.66 ± 0.14	0.63 ± 0.05	0.82 ± 0.05	0.80 ± 0.13
PCS1	**Control**	1.00 ± 0.13	1.00 ± 0.12	1.00 ± 0.07	1.00 ± 0.17
	5 µM Cd	2.71 ± 0.04	2.90 ± 0.17	1.44 ± 0.16	1.51 ± 0.19
PCS2	**Control**	1.00 ± 0.13	1.00 ± 0.05	1.00 ± 0.09	1.00 ± 0.09
	5 µM Cd	0.99 ± 0.16	2.23 ± 0.17*	2.66 ± 0.15	2.28 ± 0.32
Pro-oxidative genes					
RBOHC	**Control**	1.00 ± 0.44	1.00 ± 0.37	1.00 ± 0.39	1.00 ± 0.39
	5 µM Cd	215.33 ± 106.12	14.53 ± 4.05	3.79 ± 0.26	1.86 ± 0.97
Signal transduction genes					
OXI1	**Control**	1.00 ± 0.12	1.00 ± 0.22	1.00 ± 0.06	1.00 ± 0.05
	5 µM Cd	117.77 ± 52.95	46.74 ± 2.73	12.49 ± 2.06	7.52 ± 1.27
MPK3	**Control**	1.00 ± 0.11	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.15
	5 µM Cd	4.06 ± 3.79	3.79 ± 0.15	2.38 ± 0.24	1.29 ± 0.14*
MPK6	**Control**	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.11
	5 µM Cd	1.78 ± 0.24	1.93 ± 0.12	1.04 ± 0.03	0.84 ± 0.09

Tukey's Test per time point; except for AT1G05340: non-parametric Wilcoxon rank-sum test.

Supplemental file 5.5 fresh weight, growth inhibition, Cd content & glutathione content in ein2-5 mutant plants.

*A comparison of the fresh weight biomass & growth inhibition (**A**), Cd content (**B**) and glutathione content (**C**: reduced, oxidised and total; nmol GSH equivalents g^{-1} FW) in leaves of 3-week-old wild-type and ein2-5 Arabidopsis thaliana plants exposed for 24 or 72 h to either 5 $CdSO_4$ or grown under control conditions in a hydroponic culture system. **A:** Data shows mean ± s.e. of at least 12 biological replicates. Biomass: The letters a-d represent groups with a significantly different biomass (Tukey's test: $p < 0.05$). Statistics was performed separately within each exposure time. Growth inhibition: Significance levels, * $p < 0.05$ (Tukey's test). Statistics was performed separately within each exposure time and genotype. **B** & **C**: Data are given as mean ± s.e. of at least 4 biological replicates. **B**: The letters a-c represent groups with a significantly different Cd content after treatment (Tukey's test: $p < 0.05$). nd: levels below detection limit. Statistics was performed separately within each time point. **C**: Significant Cd-induced changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$) (Tukey's test). Statistics was performed separately for reduced, oxidised and total glutathione within each time point.*

		24 h		72 h	
A		**WT**	***ein2-5***	**WT**	***ein2-5***
FW leaves	**Control**	59.94 ± 1.86a	51.63 ± 3.65ab	97.64 ± 2.56a	80.88 ± 3.36ab
	5 µM Cd	56.44 ± 1.89ab	45.38 ± 1.98b	71.73 ± 2.49b	68.64 ± 1.50b
	% inhibition	6	12	27*	15
B		**WT**	***ein2-5***	**WT**	***ein2-5***
Cd content	**Control**	nd	nd	nd	nd
	5 µM Cd	751.57 ± 60.92a	663.59 ± 62.98a	1360.20 ± 38.67b	1040.25 ± 50.42c
C		**WT**	***ein2-5***	**WT**	***ein2-5***
GSH+GSSG	**Control**	266.77 ± 28.60	312.56 ± 18.76	235.05 ± 10.04	314.08 ± 5.84*
	5 µM Cd	255.34 ± 13.96	213.58 ± 7.91	336.26 ± 41.29	283.64 ± 6.63
GSH	**Control**	247.80 ± 29.23	295.02 ± 17.27	220.06 ± 7.93	295.58 ± 5.82*
	5 µM Cd	251.19 ± 14.43	207.98 ± 8.26	331.05 ± 40.43	278.49 ± 6.19
GSSG	**Control**	13.67 ± 1.56	17.54 ± 2.68	15.00 ± 2.21	18.50 ± 0.62
	5 µM Cd	4.15 ± 1.46	8.83 ± 3.89	5.21 ± 0.99	5.15 ± 0.89

Supplemental file 5.6. *Transcript levels in the leaves of genes encoding ethylene responsive proteins, oxidative stress markers, glutathione synthesis enzymes, pro-oxidative enzymes and signal transduction enzymes in Arabidopsis thaliana. Transcript levels were measured using quantitative real-time PCR in leaf samples of 3-week-old wild-type or ein2-5 knockout plants exposed to 5 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± s.e. of 4 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading:* $p < 0.05$*;* $p < 0.01$ *and* $p < 0.05$*;* $p < 0.01$ *for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks (*$p < 0.05$*). Abbreviations: Supplemental file 5.1.*

		24 h		72 h	
		WT	***ein2-5***	**WT**	***ein2-5***
Ethylene and jasmonate responsive genes					
ERF1	**Control**	1.00 ± 0.33	1.00 ± 0.36	1.00 ± 0.23	1.00 ± 0.16
	5 µM Cd	308.42 ± 120.40	6.60 ± 2.79*	19.18 ± 4.08	1.65 ± 0.23*
Oxidative stress marker genes					
AT2G21640	**Control**	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04
	5 µM Cd	5.86 ± 2.41	3.69 ± 1.66	5.12 ± 1.04	3.95 ± 1.40
AT2G43510	**Control**	1.00 ± 0.06	1.00 ± 0.22	1.00 ± 0.10	1.00 ± 0.14
	5 µM Cd	29.51 ± 14.76	3.86 ± 1.54*	34.05 ± 17.63	4.22 ± 1.89*
AT1G19020	**Control**	1.00 ± 0.07	1.00 ± 0.41	1.00 ± 0.18	1.00 ± 0.10
	5 µM Cd	95.07 ± 30.46	3.87 ± 1.71*	7.58 ± 0.48	1.05 ± 0.20*
AT1G05340 np	**Control**	1.00 ± 0.05	1.00 ± 0.41	1.00 ± 0.12	1.00 ± 0.04
	5 µM Cd	58.09 ± 24.67	5.45 ± 2.53*	6.92 ± 1.30	2.38 ± 1.40
AT1G57630	**Control**	1.00 ± 0.16	1.00 ± 0.41	1.00 ± 0.20	1.00 ± 0.11
	5 µM Cd	145.08 ± 54.90	4.00 ± 1.87*	7.43 ± 0.91	1.08 ± 0.34*
Glutathione synthesis genes					
GSH1	**Control**	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.14
	5 µM Cd	1.05 ± 0.05	0.71 ± 0.07*	1.04 ± 0.19	0.92 ± 0.17
GSH2	**Control**	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.07
	5 µM Cd	2.52 ± 0.61	1.02 ± 0.04*	1.56 ± 0.25	1.26 ± 0.16
GR1	**Control**	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.09
	5 µM Cd	2.40 ± 0.48	1.38 ± 0.26	1.34 ± 0.15	0.93 ± 0.09
GR2	**Control**	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.18
	5 µM Cd	0.66 ± 0.14	0.75 ± 0.10	0.82 ± 0.05	0.99 ± 0.14
PCS1	**Control**	1.00 ± 0.13	1.00 ± 0.22	1.00 ± 0.07	1.00 ± 0.10
	5 µM Cd	2.71 ± 0.04	1.41 ± 0.10*	1.44 ± 0.16	0.73 ± 0.11*
PCS2	**Control**	1.00 ± 0.13	1.00 ± 0.11	1.00 ± 0.09	1.00 ± 0.18
	5 µM Cd	0.99 ± 0.16	0.96 ± 0.12	2.66 ± 0.15	1.31 ± 0.10*
Pro-oxidative genes					
RBOHC	**Control**	1.00 ± 0.44	1.00 ± 0.67	1.00 ± 0.39	1.00 ± 0.36
	5 µM Cd	215.33 ± 106.12	6.11 ± 3.40*	3.79 ± 0.26	1.28 ± 0.57
Signal transduction genes					
OXI1	**Control**	1.00 ± 0.12	1.00 ± 0.40	1.00 ± 0.06	1.00 ± 0.22
	5 µM Cd	117.77 ± 52.95	13.40 ± 8.39*	12.49 ± 2.06	3.36 ± 0.75*
MPK3	**Control**	1.00 ± 0.11	1.00 ± 0.17	1.00 ± 0.10	1.00 ± 0.13
	5 µM Cd	4.06 ± 0.39	1.63 ± 0.29*	2.38 ± 0.24	0.73 ± 0.08*
MPK6	**Control**	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.10	1.00 ± 0.10
	5 µM Cd	1.78 ± 0.24	1.18 ± 0.16*	1.04 ± 0.03	0.78 ± 0.09

Tukey's Test per time point; except for AT1G05340: non-parametric Wilcoxon rank-sum test.

Chapter 6

General discussion & future perspectives

6.1 Study outline

Reduced crop production and economic losses caused by elevated toxic metal concentrations in the environment originating from anthropogenic activities such as mining, smelting and the use of metal-containing fertilisers and pesticides are a worldwide problem. Contamination with toxic metals, like the non-essential metal cadmium (Cd), can cause serious problems to all organisms, even when present in trace amounts. Moreover, the bioaccumulation of Cd in the food chain poses a great treat to the public health (Clemens et al., 2013). In plants, Cd disturbs several physiological and developmental processes. Despite its non redox-active character, Cd is also capable of inducing the production of reactive oxygen species (ROS) at the cellular level thereby disturbing the redox balance, the delicate balance between pro- and antioxidants (Cuypers et al., 2012; Dalcorso et al., 2010). Increasing evidence suggests a link between redox processes and the phytohormone ethylene in control of defence responses to various (a)biotic stresses. Stress-mediated ethylene induces the oxidative burst and affects the biosynthesis of glutathione (GSH), an important chelating agent and antioxidant in controlling the Cd-induced oxidative challenge (Mersmann et al., 2010; Montero-Palmero et al., 2014b; Yoshida et al., 2009). Increasing our knowledge about cellular and molecular stress signalling is necessary to further understand the plant responses to toxic Cd exposure.

The main objective of this study was to unravel the link between ethylene biosynthesis and signal transduction on one hand, and the Cd-induced oxidative challenge in *Arabidopsis thaliana* on the other hand. Plants were exposed to sublethal and environmentally realistic Cd concentrations (5 & 10 µM Cd). In a first part, the mechanistic basis of the effect of short-term Cd exposure (24 & 72 h) on the ethylene biosynthesis was investigated since previous research only determined the effect of Cd on the ethylene production levels (Chapter 3). Subsequently, the long-term impact of Cd-induced ethylene production on the ability of continuously exposed *A. thaliana* plants to complete their life cycle as well as the short-term responses on molecular and metabolic oxidative stress

parameters were determined in roots and leaves of WT and *acs2-1acs6-1* double KO-mutant plants. This mutant lacks 2 isoforms of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) enzymes (ACS2 and ACS6) that are stress-sensitive (Chapter 4). Finally, the link between ethylene signalling and the oxidative challenge induced by moderate Cd concentrations (5 µM Cd) was investigated in the leaves of different *A. thaliana* mutants with an impaired ethylene signal transduction pathway. Short-term responses of the cellular redox balance were investigated at molecular and metabolic levels, special attention was given to the GSH metabolism.

Arabidopsis thaliana, an important model organism in plant science was used during all experiments of this study. Although not of major agronomic significance, *A. thaliana* offers many important advantages for molecular research. Its small genome of 125 megabases has been sequenced and annotated, and numerous mutant and transformant lines are accessible. Moreover, extensive genetic and molecular databases and tools are available. Finally, *A. thaliana* also has a relatively short life cycle and is easily cultivated (The Arabidopsis Information Research). In our research group, Smeets et al. (2008) developed a hydroponic culture system in which controlled *A. thaliana* plant growth was optimised.

6.2 Cadmium-induced oxidative stress and ethylene production in leaves and roots of wild-type *Arabidopsis thaliana* plants

It is well-documented that the phytotoxicity of Cd is closely related to the production of ROS, disturbing the cellular redox state (Cuypers et al., 2011; Remans et al., 2010). In this study, the oxidative challenge induced by environmentally realistic Cd concentrations (5 & 10 µM; Krznaric et al., 2009), of which the growth-inhibiting effect on roots and leaves demonstrate their phytotoxicity, was investigated in WT *A. thaliana* plants (Fig. 3.4 & 4.1). In roots, we only observed a growth inhibition after 72 h of exposure to both Cd concentrations. In leaves, acute (24 h) exposure already reduced the growth after severe stress conditions (10 µM Cd), while prolonged (72 h) exposure to both Cd concentrations exerted a growth inhibiting effect. Important enzymatic sources of ROS production in plants are NADPH oxidases, also called respiratory

burst oxidase homologues (RBOHs), catalysing the formation of superoxide ($O_2^{\circ-}$). We measured the transcript levels of different members of the RBOH multigene family, shown to be important after Cd exposure (Cuypers et al., 2011; Remans et al., 2010; Smeets et al., 2009). In both organs exposure to Cd increased the expression of different *RBOH* genes. In accordance, the transcript levels of the different hallmark genes for oxidative stress increased after Cd exposure, indicating formation of ROS (Fig. 4.5 & 4.6, Supplemental file 4.1 & 4.2). These findings are in agreement with previous studies, confirming the Cd-induced oxidative stress.

Increased ethylene production levels after exposure to Cd have been reported multiple times (reviewed by Chmielowska-Bąk et al., 2014). However, the underlying mechanism of this Cd-induced increase has not been explored to date. We observed increased accumulation of free as well as conjugated ACC in roots and leaves of WT plants after exposure to both concentrations of Cd (Fig. 3.1). The overall ACC content was lower in roots compared to leaves, which can be explained by different mechanisms. Firstly, since the production of ACC by ACS covers the rate-limiting step of the ethylene biosynthesis, most of the ACC could immediately be converted into ethylene by ACO. The fact that exposure to 5 µM Cd did not significantly increase the ACC content in roots, while the expression of ethylene responsive genes did increase, supports this statement. Secondly, ACC could be transported from the roots to the shoots, serving as a messenger to promote ethylene production in the leaves. Earlier results by Jackson (1997) also observed the requirement of ACC transport from flooded roots to the leaves to raise ethylene production in the latter.

Increased ethylene biosynthesis was evident from increased expression of genes known to be responsive to elevated ethylene levels (Vandenbussche et al., 2012) in the roots and leaves of hydroponically cultivated WT plants exposed to 5 or 10 µM Cd (Fig. 3.5). This was confirmed by measuring the ethylene, albeit in a different cultivation system (rockwool). To compare the results of both systems, we corrected for Cd uptake (Table 3.1). Acute and prolonged exposure to Cd increased the ethylene emission in a dose-dependent manner in WT plants (Fig. 3.3). To further unravel the mechanistic basis of these findings, the expression of the genes encoding the enzymes involved in ethylene biosynthesis, ACS and ACO were analysed. The transcript levels of the *ACO*

multigene family, especially *ACO2* and *ACO4*, increased in a dose-dependent manner, with a maximum after 24 h exposure (Fig. 3.2). Earlier research concluded that upregulation of *ACO* genes serves as a good ethylene production indicator (Ruduś et al., 2012). Nevertheless, the production of ACC by ACS covers the rate-limiting step in the ethylene biosynthesis pathway. The transcript levels of the *ACS* multigene family reached a maximum after 72 h Cd exposure in roots and after 24 h in leaves (Fig. 3.2), supporting the ACC measurement in both organs (Fig. 3.1). Exposure to Cd particularly increased the abundance of the transcript levels of two *ACS* isoforms, *ACS2* and *ACS6*. These isoforms are the only active type 1 ACS proteins and they often appear to regulate the production of stress-ethylene in *A. thaliana*. Type 1 ACS proteins can be phosphorylated by mitogen-activating protein kinase (MAPK) MPK3/MPK6, prolonging their half-life. In addition, MPK3 and MPK6 induce the transcription of both *ACS2* and *ACS6* (Li et al., 2012; Lin et al., 2009; Skottke et al., 2011). Previous research showed increased activity and mRNA levels of MPK3 and MPK6 after exposure to Cd. Opdenakker et al. (2012) concluded that Cd-induced oxidative stress leads to altered *MPK3/6* transcript levels and Liu et al. (2010) reported that cadmium activates MPK3/6 through accumulation of ROS. Taken together, these results indicate the existence of a link between the Cd-induced oxidative challenge and ethylene production.

6.3 The spatiotemporal effects of Cd-induced ethylene biosynthesis on plant growth in *Arabidopsis thaliana* plants

Exposure to Cd increased the ethylene production in WT *A. thaliana* plants. Since ACS enzymes cover the rate-limiting step of the ethylene biosynthesis and Cd particularly affected the transcript levels of *ACS2* and *ACS6*, the Cd-induced ethylene production was measured in plants lacking both ACS2 and ACS6: double knockout (KO) *acs2-1acs6-1* mutants. A much lower Cd-induced ethylene production was observed in these mutants compared to the WT plants (Fig. 3.3). Due to the presence of the other ACS isoforms, residual ethylene production was measured. Consequently, the expression of the ethylene responsive genes was also reduced in the *acs2-1acs6-1* mutant plants, although after exposure to severe Cd concentrations (10 µM) elevated transcript levels were observed (Fig.

3.5). Next to the presence of other ACS isoforms, this increase in expression of the ethylene responsive genes could be due to the activation of other signalling pathways after severe Cd exposure, e.g. the expression of ETHYLENE RESPONSE FACTOR1 (*ERF1*) was also shown to be responsive to another phytohormone, jasmonate (Lorenzo et al., 2003). Furthermore, after 72 h exposure to Cd, the differences in expression of the ethylene responsive genes between both genotypes started to fade. This indicates a transient ethylene response, as observed by Montero-Palmero et al. (2014a) in mercury (Hg) treated *A. thaliana* seedlings.

It is known that several ACS isoforms co-ordinately generate the basal ethylene levels in *A. thaliana* (Tsuchisaka et al., 2009; Vandenbussche et al., 2012). In agreement, Skottke et al. (2011) observed similar basal ethylene production levels in the WT and *acs2-1acs6-1* mutant *A. thaliana* plants. Consequently, we did not observe phenotypic differences between the WT and the *acs2-1acs6-1* mutant plants under control conditions (Fig. 3.4, 4.1, 4.2 & 4.3). After exposure to Cd, no enhanced ethylene production was observed in these mutants in contrast to the WT plants. Therefore, short- (24 & 72 h) and long-term (up to 40 days) growth responses after moderate (5 µM) and more severe (10 µM) Cd exposure were investigated in WT and *acs2-1acs6-1* mutant plants. No differences in Cd uptake were observed in the roots and the leaves between both genotypes, implying that differences in subsequently investigated parameters solely arose from the lack of the Cd-induced ethylene production in the *acs2-1acs6-1* mutants (Fig. 4.1). In the roots, exposure to Cd did not induce phenotypic differences between both genotypes. However, the differences in leaf fresh weight after exposure to 5 µM Cd clearly revealed that the *acs2-1acs6-1* mutant plants were less sensitive to Cd compared to the WT plants (Fig. 3.4). Moreover, it supports the stress-responsiveness of the ACS2 and ACS6 isoforms. Previous research already showed that ethylene potentially inhibits the growth of plants (Dugardeyn and Van Der Straeten, 2008). In the long-term experimental setups however, Cd exposure did not induce phenotypic differences between both genotypes in the roots and the leaves, again demonstrating the early and transient role for ethylene in the response to Cd stress (Fig. 4.2 & 4.3). Also with regard to reproduction, both the chronically exposed WT and *acs2-1acs6-1* mutant plants were capable of producing as much germinative seeds (Fig. 4.4).

Stress severity determines which signal-sensing systems are activated. Previous results investigating ethylene responsive genes and leaf fresh weight demonstrate that most differences between the WT and *acs2-1acs6-1* mutant plants are observed after exposure to moderate (5 µM) Cd stress (Fig. 3.4, 3.5 & 4.1). We therefore hypothesise that severe (10 µM) Cd stress overwhelms the plants, activating different signal-sensing systems that potentially bypass the ethylene signal. The expression of jasmonate responsive genes such as *VSP2* increased in *acs2-1acs6-1* mutant plants in a dose dependent way that was absent in the WT plants indicating a role for jasmonates as ethylene bypass signals (Fig. 4.6 E, Supplemental file 4.2). Consistently, various studies revealed a link between ethylene and jasmonate signalling, suggesting that they can act both synergistically, antagonistically and interdependently of each other (Lorenzo et al., 2003; Wasternack, 2007; Zhu, 2014). Moreover, the cross-talk between ethylene and various other phytohormone signalling pathways (e.g. gibberellin, abscisic acid, auxin, etc.) under (a)biotic stress conditions has also been shown, suggesting they could all have a role in potential ethylene bypass signals (Achard et al., 2006; Anderson et al., 2004; Dugardeyn and Van Der Straeten, 2008; Vandenbussche et al., 2010).

Given that ethylene is predominantly involved in the early responses to moderate Cd stress in the leaves, we increased our experimental resolution to these findings. The growth differences between WT and the *acs2-1acs6-1* mutant plants were confirmed using different ethylene insensitive mutants, covering distinct steps in the ethylene signal transduction pathway, ETHYLENE RESISTANT1 (receptor; *etr1-1*), ETHYLENE INSENSITIVE2 (signal transducer; *ein2-1*) and ETHYLENE INSENSITIVE3 (transcription factor; *ein3-1*). Unexposed *etr1-1* and *ein2-1* mutant rosettes were significantly smaller compared to WT and *ein3-1* mutant rosettes (Fig. 5.1 A & B). However, exposure to Cd inhibited the rosette growth of the latter (WT, *ein3-1* mutants), while this inhibition was absent in the other two genotypes (Fig. 5.1 A & B). The dissimilarities between *ein3-1* and the other 2 mutant genotypes can be explained by the fact that *ein3-1* is less ethylene insensitive due to the existence of EIN3-LIKE (EIL) transcription factors (Chao et al., 1997). These results indicate that, although ethylene signalling is necessary for normal growth, increased Cd-induced stress-ethylene production and signalling has a negative effect on rosette development.

6.4 The Cd-induced oxidative challenge is mediated by ethylene biosynthesis and signalling in *Arabidopsis thaliana* plants

We concluded that Cd induces (1) the production of ethylene (Schellingen et al., 2014) and (2) oxidative stress in WT *A. thaliana* plants. Different studies reported that ethylene increases oxidative stress by inducing the oxidative burst through the activation of NADPH oxidases (Mersmann et al., 2010; Montero-Palmero et al., 2014a). Previous research in our group identified RBOHC as an important NADPH oxidase isoform of which the expression was induced by Cd exposure in WT *A. thaliana* leaves (Cuypers et al., 2011; Remans et al., 2010; Smeets et al., 2009). Except for the *ein3-1* mutant, the expression of *RBOHC* was lower in the different mutants compared to WT plants (Fig. 4.6 C & 5.2 B). In accordance, the expression of the five oxidative stress hallmark genes described by Gadjev et al. (2006) increased in the WT and *ein3-1* mutant plants, which was less in the *etr1-1* mutants and overall lower in the *acs2-1acs6-1* and *ein2-1* mutants compared to the WT plants (Fig. 4.6 A & B, 5.2 B; Supplemental file 4.2, 5.2 & 5.3). Most of these differences were observed after 24 h Cd exposure, while prolonged exposure diminished the differences between the WT and mutant plants. These results indicate the early and transient role of ethylene in the Cd-induced oxidative challenge, since *acs2-1acs6-1* lacks the Cd-induced ethylene production and *ein2-1* is the only complete ethylene insensitive mutant.

Glutathione is an important metabolite in Cd-induced responses due to its dual role. Firstly, it is an important chelator of Cd due to the high affinity of Cd for the thiol group of GSH and as the precursor for phytochelatins. Secondly it also serves multiple functions in the antioxidative defence system (Jozefczak et al., 2012; Sobrino-Plata et al., 2014). Moreover, the existence of a cross-talk between ethylene and the GSH metabolism has been revealed in various studies (Cao et al., 2009; Masood et al., 2012; Yoshida et al., 2009). Exposure to Cd increased the expression of glutathione synthetase (*GSH2*) and glutathione reductase (*GR1*) in WT *A. thaliana* plants (Fig. 4.6 G & H, 5.2 A; Supplemental file 4.2, 5.2 & 5.3). The expression of these genes was shown to be important in alleviating the Cd- and ozone-induced depletion of GSH (Yoshida et al., 2009; Zhu et al., 1999). Correspondingly, the levels of reduced and total GSH remained at a steady state after 24 h and even increased after 72 h Cd exposure

in WT plants (Table 4.1, 5.2). This Cd-induced increase in GSH content was already observed in the leaves of *A. thaliana* and *Brassica juncea* (Jozefczak et al., 2014; Masood et al., 2012). In the *acs2-1acs6-1* and the different ethylene insensitive mutant plants, the expression of *GSH2* and *GR1* was significantly lower compared to the WT plants (Fig. 4.6 G & H, 5.2 A; Supplemental file 4.2, 5.2 & 5.3). This resulted in a Cd-induced decrease in reduced and total GSH content in all the mutants except *ein3-1* after 24 h exposure. Prolonged (72 h) exposure restored the GSH content in these mutants although the Cd-induced increase observed in the WT plants was still absent (Table 4.1 & 5.2). In agreement, Yoshida et al. (2009) observed a decreased GSH content in ozone-exposed *ein2-1* mutant plants as a result of a suppressed *GSH2* and *GR1* expression and a suppressed and delayed GSH2 activity. Our results display a delayed GSH response in the mutants with an impaired ethylene biosynthesis or signal transduction pathway. Therefore we hypothesise the early involvement of ethylene in the GSH metabolism after Cd exposure.

In conclusion, our data show that ethylene is involved in fine-tuning the early Cd-induced oxidative challenge in *Arabidopsis thaliana* leaves exposed to moderate Cd concentrations (Fig. 6.1).

6.5 Future perspectives

We uncovered a link between ethylene and oxidative stress in the early response of *Arabidopsis thaliana* leaves to moderate Cd exposure (Fig. 6.1). Although diminished transcript levels of oxidative stress hallmark genes were measured in mutant plants with impaired ethylene production or signalling under Cd exposure (Supplemental file 4.2, 5.2 & 5.3), further investigation of the oxidative stress profile will increase our knowledge concerning the underlying mechanisms of the cross talk between ethylene and oxidative stress. Measuring the activities of different pro-oxidative (NADPH oxidases, lipoxygenases (LOX)) and antioxidative (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), etc.) enzymes or the concentration of different ROS ($O_2^{\circ-}$, H_2O_2) could serve as a basis in future research (Cuypers et al., 2011).

The early involvement of ethylene in the GSH metabolism also arose from our data. To further unravel this link, the concentration of PCs, serving as Cd

chelators, should be measured. Jozefczak et al. (2014) observed an increased PC content in Cd-exposed *A. thaliana* leaves. As a precursor of PCs, the different GSH levels observed between the WT and mutant plants after Cd exposure, potentially generates differences in PC concentrations (Table 4.1 & 5.2). Elucidating this link in the roots might also be important since Jozefczak et al. (2014) observed a Cd-induced depletion of GSH levels due to allocation to PC production after 2 h Cd exposure, compromising its antioxidant role.

Ethylene is involved in many processes during the entire life cycle of the plants (Vandenbussche et al., 2012). No phenotypic differences between WT and *acs2-1acs6-1* mutant plants were observed after long-term exposure to Cd (Fig. 4.2, 4.3 & 4.4). However, the reproductive capacity of the ethylene insensitive mutant plants remains to be investigated. Due to the complete ethylene insensitivity of the *ein2-1* mutants, this could be very interesting. Furthermore, short-term exposure to severe Cd concentrations activated mechanisms bypassing the ethylene signal. We hypothesised the existence of a cross-talk between ethylene and different phytohormones, especially oxylipins such as jasmonates, as potential bypass mechanism. In order to test this hypothesis, the content of different oxylipins in Cd-exposed WT and mutant *A. thaliana* plants with an impaired ethylene biosynthesis or signalling should be measured (Grebner et al., 2013).

To summarise, many aspects of the link between the Cd-induced oxidative challenge and ethylene biosynthesis/signalling should be further elucidated. Our results indicate both *acs2-1acs6-1* and *ein2-1* as most promising mutants for this purpose in future experiments.

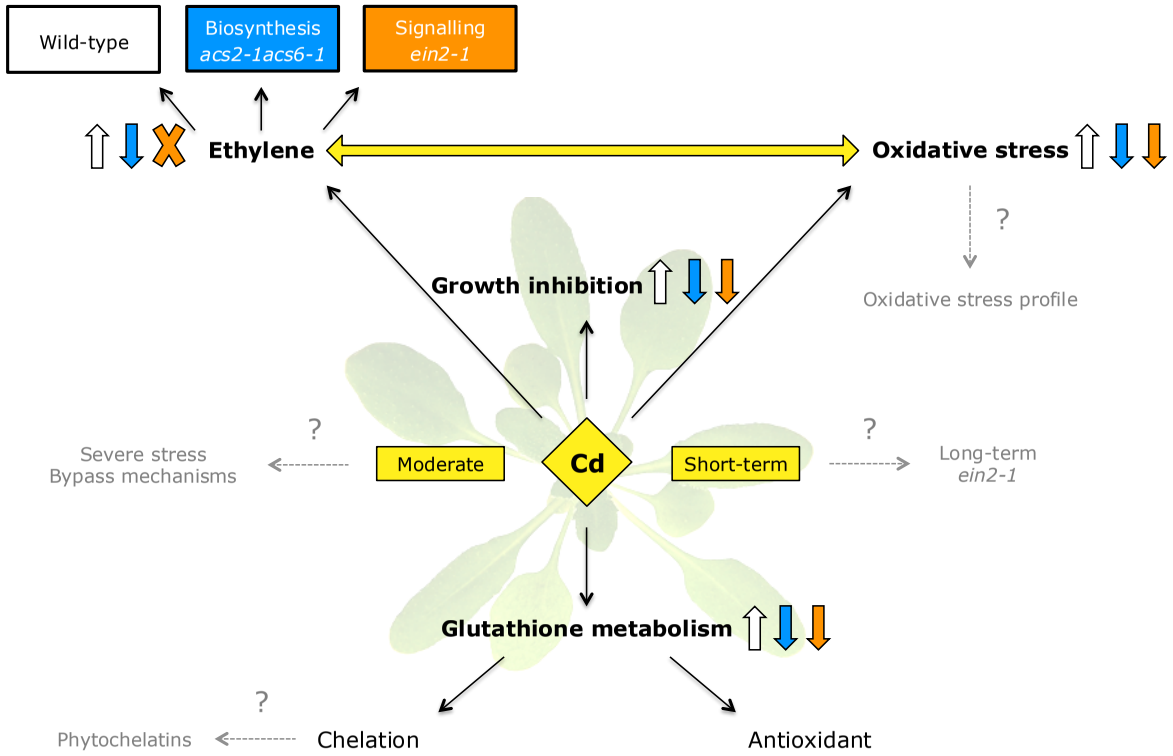

Figure 6.1 *Schematic overview of the proposed model displaying the link between the oxidative challenge and ethylene after short-term exposure to moderate Cd concentrations in Arabidopsis thaliana leaves. White arrows show cadmium-induced responses in WT plants. Exposure to Cd increases ethylene production, oxidative stress and glutathione biosynthesis, together resulting in growth inhibition. Genotypic differences compared to the WT plants due to impaired ethylene production or signalling are shown in blue (acs2-1acs6-1) or orange (ein2-1). Grey arrows and text represent interesting future perspectives (See main text for further details). ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC Synthase; Cd, cadmium; EIN, ethylene insensitive.*

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BOOK CHAPTER

Cuypers, A., Keunen, E., Bohler, S., Jozefczak, M., Opdenakker, K., Gielen, H., Vercampt, H., Bielen, A., Schellingen, K., Vangronsveld, J. and Remans, T. (2012). Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling. In D. Gupta, L. M. Sandalio, eds. *Metal Toxicity in Plants: Perception, Signaling and Remediation*. Springer-Verlag.

ABSTRACTS

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