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DOCTORAL DISSERTATION

Glutathione as key regulator of early responses to cadmium stress in *Arabidopsis thaliana*

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Summary

Samenvatting

A short summary of my experimental highlights.

Summary

Both historical and today's industrial and agricultural activities contribute to the current worldwide pollution with toxic metals such as cadmium (Cd). Bioaccumulation of Cd poses a serious threat to the food chain, and therefore the entire ecosystem including human, animal and plant populations. Plants exposed to Cd suffer from disturbances in morphology (e.g. reduced crop yield) and physiology (e.g. photosynthesis). At the cellular level, Cd elicits an oxidative challenge, characterised by an elevated production of reactive oxygen species (ROS). Increased ROS levels have been associated with both damaging oxidising processes and protective signalling. The latter is suggested to activate the antioxidant system, which neutralises ROS and hence restores the redox equilibrium. This balance between ROS production and scavenging determines whether the oxidative challenge will lead to damage or signalling. Three major redox buffers in plant cells are involved in both signalling and antioxidant processes. These redox buffers consist of the reduced and oxidised forms of glutathione [*i.e.* glutathione (GSH) and glutathione disulfide (GSSG)], ascorbate [*i.e.* ascorbate (AsA) and dehydroascorbate (DHA)] and NADP (*i.e.* NADPH and $NADP^+$, respectively). Together with enzymes, these redox buffers constitute the AsA-GSH cycle, which is essential for the plant's normal metabolism as well as its defence against oxidative stress. Under normal conditions, cells maintain highly reduced pools of these metabolites. Both GSH and AsA also have direct ROS scavenging capacity and serve as a substrate in other ROS detoxifying mechanisms. Besides these metabolites and their related enzymes, the superoxide ($O_2^{\bullet -}$)-reducing superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2)-neutralising catalase (CAT) are two major substrate-independent enzymes that complement the antioxidant system. In order to limit Cd-induced oxidative stress, plants additionally stimulate their chelation capacity. A primary Cd chelator that scavenges free damaging Cd ions, is phytochelatin (PC), a polymerised form of GSH. The thiol group on the cysteine residue of GSH causes this metabolite to function as signalling agent, antioxidant and Cd chelator.

The current work focuses on GSH as well as AsA because they are both abundant, multifunctional and widely distributed metabolites in plant cells. The main goal of this research was to expand our knowledge concerning the role of GSH and AsA as chelator, antioxidant and/or redox buffer in *Arabidopsis thaliana*

responses during moderate and severe, *i.e.* ranging from 1 to 10 µM, Cd exposure. To reveal spatiotemporal functions of GSH and AsA in early Cd detoxification, a kinetic screening was performed in wild-type plants. Cadmium chelation and antioxidative defences were investigated at different functional levels (gene expression, enzyme activity and metabolite content). The roots showed a fast but GSH-depriving PC production upon 2 h of Cd exposure. Despite SOD regulation, which already started after 2 h, alternative pathways including AsA and CAT were only activated after 24 h in order to complement the antioxidant function of GSH. Retention and detoxification of Cd in the roots contributed to a delayed response in the leaves. Together with high leaf GSH and AsA levels and possibly root-to-shoot signalling responses, the leaves had sufficient time to activate their defence mechanisms, preventing GSH deprivation. This experiment suggests a dual time shift, both within roots and between plant organs (Chapter 3). The use of multiple Cd concentrations (5 and 10 µM) and exposure times (2, 24, 48 and 72 h), provided the basis for more in-depth research conducted in the following experiments.

The use of *A. thaliana* as model organism, created the possibility to include mutants deficient in GSH and/or AsA. To gain more insight in the importance of these metabolites during the Cd-induced oxidative challenge, responses of wild-type plants were compared to mutants with 55 to 70% GSH reduction (*cad2-1*), 50 to 60% AsA reduction (*vtc1-1*) or both (*cad2-1 vtc1-1*) upon exposure to Cd during 24 (Chapter 4) and 72 h (Chapter 5). Both genotype- and Cd-specific responses concerning chelating (PC levels) and antioxidative mechanisms (gene expression, enzyme activity, metabolite redox state and content) were investigated. First, *vtc1-1* mutants revealed an increased defence capacity under control conditions, including thiols (GSH and PC) and antioxidant transcripts [monodehydroascorbate reductase 3 (*MDAR3*) and glutaredoxin 480 (*GRX480*)]. In addition, these mutants did not demonstrate elevated oxidative stress at the level of GSH and AsA redox state or oxidative stress marker genes. Upon Cd exposure, enhanced PC synthesis appeared to efficiently compensate low AsA levels, contributing to a less Cd-sensitive genotype. Together, these findings suggest that AsA deficiency continuously primes *vtc1-1* plants and therefore, prepares them to cope with additional stresses like exposure to Cd. Secondly, *cad2-1* mutants were unable to compensate for their low metabolite levels.

Under control conditions, these mutants revealed a more oxidised cellular GSH redox state and elevated expression of oxidative stress marker genes. The severe lack in GSH and PC levels resulted in a higher sensitivity to Cd in *cad2-1* plants. The latter was indicated by Cd-induced activation of SOD, AsA and CAT pathways, which occurred to a greater extent in the *cad2-1* mutants than wild-type plants. The current work suggests that GSH deficiency causes initial oxidative stress and the activation of multiple alternative pathways in order to cope with additional Cd stress.

Based on data from the previous chapters, the role of GSH in SOD expression upon Cd exposure was investigated in a more in-depth experimental set-up (Chapter 6). Three mutants with GSH contents ranging from 15 to 45% of wild-type levels (*cad2-1*, *pad2-1* and *rax1-1*) and wild-type plants were exposed to 5 µM Cd during 24 and 72 h and SOD expression was evaluated in all genotypes at transcriptional, posttranscriptional and enzyme activity level. In Cd-exposed wild-type plants, the activation of the squamosa promoter-binding protein-like 7 (SPL7) transcription factor resulted in up-regulation of FeSOD1 (*FSD1*) and microRNA398 (*MIR398*), which in its turn repressed CuZnSOD (*CSD1* and *CSD2*) transcripts. Similar to wild-type plants, SPL7-dependent responses were activated in all Cd-exposed mutants, with the exception for both mature and primary *CSD1/2* transcripts, which were significantly up-regulated in all GSH deficient mutants after 24 h. This fast but transient Cd-induced transcriptional activation of *CSD* transcripts after 24 h overruled the *MIR398*-regulated repression of *CSD* expression and resulted in increased SOD activity. This study suggests that the (subcellular) thiol redox state (concentration and/or reduced-oxidised ratio) contributes to the transcription rate of *CSD* genes upon Cd exposure. Although the underlying mechanism remains unknown, a still-unidentified transcriptional activator was put forward to be responsible for this regulation.

Finally, the importance of a PC-mediated response was investigated during long-term exposure to Cd (Chapter 7). Therefore, Cd and PC accumulation were followed in wild-type plants during three weeks of Cd exposure. Plant GSH and PC to Cd molar ratios (SH/Cd) initially increased, but after three days of Cd exposure, the ratios seemed to decrease over time. Also GSH deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) were included to investigate the necessity of Cd-

induced PC production for plant survival. Despite their increased Cd sensitivity, like the wild-type plants, all mutants were able to survive. These data suggest that after a fast PC response, including both Cd chelation and compartmentalisation, plants activate alternative mechanisms that complement the early Cd detoxification processes.

In conclusion, all results support the essential role for GSH in early responses to Cd exposure. Both Cd chelation via PCs and ROS scavenging via SOD appeared to be a primary response in plants. Therefore, the lack of GSH and hence PCs, resulted in elevated activation of alternative antioxidant mechanisms including SOD, AsA and CAT. Moreover, this study suggests a regulatory role for GSH in SOD expression during Cd exposure, which might involve a still-unknown transcriptional activator. Long-term exposure to Cd however, indicated that the fast PC response was efficiently complemented by an alternative Cd detoxification mechanism.

Samenvatting

Zowel historische als hedendaagse antropogene activiteiten in de industrie en landbouw dragen bij aan de huidige wereldwijde vervuiling van de omgeving met cadmium (Cd). Bioaccumulatie van dit metaal vormt een ernstige bedreiging voor de voedselketen en bijgevolg voor het hele ecosysteem. Planten die blootgesteld worden aan Cd vertonen verstoringen in hun morfologie (bv. verminderde opbrengst) en fysiologie (bv. fotosynthese). Op cellulair niveau veroorzaakt Cd een verstoring in de redoxbalans, die gekenmerkt wordt door een verhoogde productie van reactieve zuurstofvormen (ROS). Verhoogde ROS concentraties worden geassocieerd met zowel schadelijke oxiderende processen als beschermende signalisatie. Deze signalisatie kan resulteren in de activatie van het antioxidatief systeem dat ROS neutraliseert en dus de redoxbalans herstelt. Het evenwicht tussen de productie en het wegvangen van ROS bepaalt of er schade of signalisatie zal optreden. Plantencellen bevatten drie belangrijke redoxbuffers die betrokken zijn in zowel signalisatie als antioxidatieve processen. Deze buffers bestaan uit de gereduceerde en geoxideerde vormen van glutathion [*i.e.* glutathion (GSH) en glutathion disulfide (GSSG)], ascorbaat [*i.e.* ascorbaat (AsA) en dehydroascorbaat (DHA)] en NADP (*i.e.* NADPH en $NADP^+$). In samenwerking met specifieke enzymen, vormen deze buffers de AsA-GSH cyclus, die essentieel is voor het normaal metabolisme alsook voor de verdediging van planten tegen oxidatieve stress. Onder controle omstandigheden komen deze metabolieten voornamelijk in gereduceerde toestand voor in cellen. Zowel GSH als AsA kunnen ROS rechtstreeks neutraliseren maar dienen tevens als substraat in andere antioxidatieve mechanismen. Naast deze metabolieten en hun gerelateerde enzymen, bestaan er twee belangrijke substraatonafhankelijke enzymen die het antioxidatief verdedigingssysteem aanvullen. Enerzijds zorgt superoxide dismutase (SOD) voor de reductie van superoxide ($O_2^{\bullet -}$) en anderzijds neutraliseert catalase (CAT) waterstofperoxide moleculen (H_2O_2). Om oxidatieve stress ten gevolge van Cd te beperken, stimuleren planten bovendien hun capaciteit om Cd rechtstreeks te binden via chelators. Fytochelatine (PC) is een gepolymeriseerde vorm van GSH en tevens een primaire chelator die vrije en schadelijke Cd ionen bindt. Glutathion dankt zijn functies als signaalmolecule, antioxidant en Cd chelator aan de thiol groep op zijn cysteineresidu.

De huidige studie focust zich op zowel GSH als AsA omdat het beide abundante en multifunctionele metabolieten zijn met een brede verspreiding in plantencellen. Het hoofddoel van dit onderzoek is het uitbreiden van onze kennis omtrent de rol van GSH en AsA als chelator, antioxidant en/of redoxbuffer in reacties van *Arabidopsis thaliana* tijdens gematigde en hogere Cd blootstelling, *i.e.* tussen 1 en 10 µM Cd. Om de functies van GSH en AsA tijdens vroege Cd detoxificatie zowel in tijd en ruimte te onderzoeken, werd een kinetische screening uitgevoerd in wild-type planten. Cadmium chelatie en antioxidatieve verdedigingsmechanismen werden bestudeerd op verschillende functionele niveaus (genexpressie, enzymactiviteit en metabolietgehalte). De wortels vertoonden een snelle maar GSH-consumerende productie van PCs na 2 u blootstelling aan Cd. De SOD regulatie werd tevens na 2 u Cd blootstelling geactiveerd, maar andere mechanismen, zoals AsA en CAT, die eveneens de antioxidatieve functie van GSH kunnen aanvullen werden pas na 24 u gestimuleerd. Chelatie en detoxificatie van Cd in de wortels draagt bij tot een vertraagde reactie in de blaadjes. Samen met hoge gehalten aan GSH en AsA in het blad en mogelijke signalisatie vanuit de wortel, werden de verdedigingsmechanismen in de blaadjes geactiveerd zonder dat er een daling in GSH gehalten optrad. Dit experiment suggereert een dubbele tijdsverschuiving, zowel binnen wortels als tussen plantorganen (Hoofdstuk 3). Het gebruik van meerdere Cd concentraties (5 en 10 µM) en blootstellingtijden (2, 24, 48 en 72 u) vormde de basis voor meer diepgaand onderzoek dat werd uitgevoerd in de volgende experimenten.

Dankzij het gebruik van *A. thaliana* als modelorganisme is er de mogelijkheid om mutanten in de studie te betrekken met een tekort aan GSH en/of AsA. Om meer inzicht te verwerven in het belang van deze metabolieten tijdens de oxidatieve stress ten gevolge van Cd blootstelling, werden mutanten gebruikt die deficiënt zijn in GSH (55 tot 70% daling; *cad2-1*), in AsA gehalten (50 tot 60% daling; *vtc1-1*) of beide (*cad2-1 vtc1-1*). De responsen in wild-type planten werden vergeleken met deze van de mutanten na een blootstelling aan Cd gedurende 24 (Hoofdstuk 4) en 72 u (Hoofdstuk 5). Zowel genotype- als Cd-specifieke reacties omtrent chelatie (PC gehalten) en antioxidatieve mechanismen (genexpressie, enzymactiviteit, metaboliet redoxbalans en gehalten) werden bestudeerd. Als eerste vertoonden *vtc1-1* mutanten een

verhoogde verdedigingscapaciteit onder controle omstandigheden, die bestond uit verhoogde thiol concentraties (GSH en PC) en antioxidatieve transcripten [monodehydroascorbaat reductase 3 (*MDAR3*) en glutaredoxine 480 (*GRX480*)] ten opzichte van wild-type planten. Bovendien vertoonden deze mutanten geen verhoogde oxidatieve stress op het niveau van de GSH en AsA redoxbalans of oxidatieve stress-gerelateerde genen. Na blootstelling aan Cd bleek een verhoogde productie van PCs een efficiënte compensatie te zijn voor de lage AsA concentraties, hetgeen bijdraagt aan een genotype dat minder gevoelig is voor Cd. Al deze bevindingen suggereren dat de *vtc1-1* planten continu geprimed worden door hun AsA deficiëntie en bijgevolg voorbereid zijn om met bijkomende stresssituaties zoals Cd blootstelling om te gaan. *Cad2-1* mutanten waren daarentegen niet in staat om hun lage GSH concentraties te compenseren. Onder controle omstandigheden vertoonden deze mutanten bovendien een meer geoxideerde cellulaire GSH redoxstatus en een verhoogde expressie van oxidatieve stress-gerelateerde genen. Het tekort aan GSH en PC gehalten resulteerde in een hogere gevoeligheid van *cad2-1* planten voor Cd blootstelling. Dit laatste werd geassocieerd met een Cd-afhankelijke activatie van SOD, AsA en CAT mechanismen die groter was in de *cad2-1* mutanten dan in wild-type planten. Het huidige werk suggereert dat GSH deficiëntie een verhoogde initiële oxidatieve stress met zich meebrengt en de activatie van meerdere alternatieve mechanismen vraagt om met de bijkomende Cd stress om te gaan.

Op basis van data uit de voorgaande hoofdstukken, werd de rol van GSH in SOD expressie na blootstelling aan Cd bestudeerd in een meer diepgaand experiment (Hoofdstuk 6). Drie mutanten met GSH gehalten die variëren tussen 15 en 45% van wild-type concentraties (*cad2-1*, *pad2-1* en *rax1-1*) werden blootgesteld aan 5 µM Cd gedurende 24 en 72 u en vergeleken met wild-type planten op het niveau van transcriptie, posttranscriptie en enzymactiviteit. Cadmium-blootstelling in wild-type planten resulteerde in de activatie van de squamosa promoter-binding protein-like 7 (SPL7) transcriptiefactor en bijgevolg een verhoogde expressie van FeSOD1 (*FSD1*) en microRNA398 (*MIR398*), die op zijn beurt CuZnSOD (*CSD1* en *CSD2*) transcripten onderdrukte. Zoals in wild-type planten, werden SPL7-afhankelijke reacties geactiveerd in alle mutanten na Cd blootstelling, *i.e.* de inductie van *FSD1* en *MIR398*, desondanks werden zowel

mature als primaire *CSD1/2* transcripten significant verhoogd in alle GSH deficiënte mutanten na 24 u. Deze snelle maar tijdelijke transcriptionele activatie van *CSD* transcripten overtrof de *MIR398*-gereguleerde onderdrukking en resulteerde in een verhoogde SOD activiteit na blootstelling aan Cd. Deze studie suggereert dat de (subcellulaire) thiol redoxstatus (thiol concentratie en/of verhouding gereduceerd-geoxideerd) bijdraagt aan de transcriptiesnelheid van *CSD* genen tijdens blootstelling aan Cd. Hoewel het onderliggende mechanisme nog onbekend is, wordt een ongeïdentificeerde transcriptionele activator naar voren gebracht als verantwoordelijke voor deze regulatie.

Tot slot werd het belang van een PC-gemedieerde reactie bestudeerd bij langdurige blootstelling aan Cd (Hoofdstuk 7). Hiertoe werd de opstapeling van Cd en PCs gevolgd in wild-type planten bij een blootstelling aan Cd gedurende drie weken. De molaire verhouding tussen GSH en PCs enerzijds en Cd anderzijds (SH/Cd) steeg gedurende de initiële blootstelling, maar nam tijdsafhankelijk af na drie dagen. Ook GSH deficiënte mutanten (*cad2-1*, *pad2-1* en *rax1-1*) werden gebruikt in dit experiment om de noodzaak van PC productie te bestuderen voor overleving in planten na blootstelling aan Cd. Ondanks hun verhoogde gevoeligheid voor Cd waren alle mutanten in staat om te overleven, net zoals de wild-type planten. Deze data suggereren dat na een snelle chelatie van Cd met PCs, gevolgd door een sekwestratie in verschillende compartimenten, planten alternatieve mechanismen activeren die de vroege processen van Cd detoxificatie aanvullen.

Ter conclusie kunnen we stellen dat alle resultaten de essentiële rol voor GSH in de vroege respons van planten op Cd blootstelling ondersteunen. Zowel Cd chelatie via PCs als ROS neutralisatie via SOD bleken primaire reacties te zijn in planten. Hierdoor resulteerde het gebrek aan GSH, en bijgevolg PCs, in een verhoogde activatie van alternatieve antioxidatieve mechanismen inclusief SOD, AsA en CAT. Bovendien toont deze studie een regulerende rol voor GSH aan in de expressie van SOD tijdens blootstelling aan Cd, hetgeen de betrokkenheid van een nog steeds onbekende transcriptionele activator suggereert. Langdurige blootstelling aan Cd daarentegen, toonde aan dat de snelle reactie met PCs efficiënt werd aangevuld door een alternatief mechanisme van Cd detoxificatie.

Table of contents

List of abbreviations

A list of abbreviations that are often used in this dissertation.

APx	Ascorbate peroxidase
AsA	Ascorbate (reduced)
CAT	Catalase
Ca	Calcium
Cd	Cadmium
$CdSO_4$	Cadmium sulfate
Cu	Copper
CuZnSOD/*CSD*	Copper/zinc superoxide dismutase (enzyme/gene)
Cys	Cysteine
DHA	Dehydroascorbate (oxidised)
DHAR	Dehydroascorbate reductase
Fe	Iron
FeSOD/*FSD*	Iron superoxide dismutase (enzyme/gene)
γ-EC	*γ*-Glutamylcysteine
GDP	Guanosine diphosphate
Glu	*γ*-Glutamate
Gly	Glycine
GME	GDP-D-mannose 3',5'-epimerase
GMP	GDP-D-mannose pyrophosphorylase
GPOD	Guaiacol peroxidase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GRx	Glutaredoxin
GSH	Glutathione (reduced)
GSH1	*γ*-Glutamylcysteine synthetase
GSH2	Glutathione synthetase
GSSG	Glutathione disulfide (oxidised)
H_2O_2	Hydrogen peroxide
K	potassium
LOX	Lipoxygenase
MDA	Monodehydroascorbate
MDAR	Monodehydroascorbate reductase
Mg	Magnesium
MIR398	MicroRNA398

Mn	Manganese
MnSOD/*MSD*	Manganese superoxide dismutase (protein/gene)
MRP	multidrug resistance-associated protein
MT	Metallothionein
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
$^3O_2$	Molecular oxygen
O_2	Singlet oxygen
$O_2^{\bullet -}$	Superoxide radical
$^{\bullet}OH$	Hydroxyl radical
PC	Phytochelatin
PCS	Phytochelatin synthase
pri-CSD	Primary copper/zinc superoxide dismutase transcripts
pri-MIR	Primary microRNA398 transcripts
PRx	Peroxiredoxin
RBOH	NADPH oxidase gene (respiratory burst oxidase homolog)
ROS	Reactive oxygen species
S	Sulfur
SH	Thiol
SOD	Superoxide dismutase
SPL	Squamosa promoter binding protein-like
SPOD	Syringaldazine peroxidase
TBArm	Thiobarbituric acid-reactive metabolites
TRx	Thioredoxin
Zn	Zinc

Chapter 1

General introduction

Review – published

Glutathione is a key player in metal-induced oxidative stress defences

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1.1. Introduction

Metals are natural components of the earth's crust; low background concentrations can be detected in soils, sediments, waters and even in organisms. Due to their versatile applicability, metals are widely used in, amongst others, electronic components, building materials, motor fuels and fertilizers. Since the industrial revolution in 1815, advanced industrial and agricultural activities have exponentially increased the production and consequently the emission of metals. Unlike many organic pollutants, which potentially degrade to carbon dioxide (CO_2) and water (H_2O), metals are not biodegradable and persist in the environment. The cumulative industrial release into our environment has been massive and has overwhelmed the natural cycles of metals in many ecosystems (Nriagu & Pacyna 1988; Nriagu 1996; Chary *et al.* 2008). Quantification of material extraction for the global economy forecasts a significant growth of resource extraction. From all resources, the extraction of metal ores has proportionally increased the most since 1980. An even larger increase is predicted for the near future, indicating the continued importance of metals for industrial development, with the consequence of increased emissions into the environment (Behrens *et al.* 2007; Giljum *et al.* 2008).

Essential metals [cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn)] serve as critical micronutrients for normal development and growth of organisms. Since these elements can become toxic at higher levels, plants have developed a strict regulation to absorb, translocate and store them within physiological ranges. Despite the selectivity of transport systems, non-essential metals and metalloids like arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb) and mercury (Hg) also make use of these uptake mechanisms (Verbruggen *et al.* 2009b). The main environmental threats are associated with these non-essential elements because plants, as primary producers, form an important entry pathway for potentially toxic substances into the food chain (Leonard *et al.* 2004; Chary *et al.* 2008). Regardless of this knowledge, metal emissions are still continuing, particularly in less developed countries (Järup 2003; Järup & Akesson 2009; Eze *et al.* 2010).

1.1.1 *Importance of cadmium research*

The substantial 100 year long emission of Cd by three Zn smelters in the Campine area across the Dutch-Belgian border resulted in soil contamination with As, Cd, Pb and Zn of a 450 km² large area. Both Zn and Pb were refined via ore smelting up to 1400°C. Metal vapours were condensed and collected, while the solid waste was used for paving roads and industrial sites. Metals that were not maintained in the condenser, precipitated on dust particles that were emitted through the chimney. In this way, metals with a relatively low boiling point like Cd (321°C) were spread throughout the environment. Additional wastewater discharge contributed to pollution of sediments (Scokart *et al.* 1983). Due to the increasing knowledge on metal toxicity, new environmental legislation and regulations led to stricter conditions, including greater control of all emissions during the smelting process. As a result, the smelter in Lommel was dismantled in 1974 and the other factories shifted from a thermal to a new and less-polluting electrolytic Zn refining process. Later, production of Cd was stopped completely in Overpelt (in 1992) and Balen (in 2002) (VMM 2013). Despite the absence of current Cd emission, historical pollution remains an environmental threat because metals are non-biodegradable. On the one hand, human activities can further distribute metal contamination, e.g. by the use of contaminated well water. On the other hand, nature continuously spreads metals via wind erosion and leaching to ground water due to heavy rain (Järup 2003).

Cadmium is of serious concern because it is easily spread throughout the environment and it has been associated with severe health problems (Nair *et al.* 2013). Exposure to Cd occurs through intake of contaminated food or drinking water, inhalation of tobacco smoke or polluted air (Hogervorst *et al.* 2007; Nordberg *et al.* 2007). The natural Cd concentrations lie between 0.1 and 0.5 mg/kg soil, but the norm is 1 mg/kg. In the Campine region however, this can add up to more than 12 mg/kg, affecting the entire ecosystem. The main metabolic feature of Cd is its exceptionally long biological half-life (10 to 30 years), which results in a virtually irreversible accumulation throughout life. The two main storage sites for Cd in humans are the liver and kidney, containing about 40 to 80% of the body burden (Järup *et al.* 1983; Nordberg *et al.* 2007). Numerous studies have associated environmental Cd exposure with disorders

including renal dysfunction (Buchet *et al.* 1990; Staessen *et al.* 1994), increased calciuria (Staessen *et al.* 1991; Nawrot *et al.* 2010), osteoporosis (Staessen *et al.* 1999; Nawrot *et al.* 2010), increased risks of fractures (Staessen *et al.* 1999), cancer (Nawrot *et al.* 2006; Järup & Akesson 2009) and mortality (Nawrot *et al.* 2008). Recent efforts in a search for biomarkers point towards the "omics" field including genomics, proteomics and metabolomics. These technologies permit early detection of cellular responses to toxic agents like Cd (Fowler 2009; Ellis *et al.* 2012). Another field of research is focused on strategies for phytoextraction (*i.e.* soil clean-up or uptake of metals from the soil into the plant biomass) and phytostabilisation (*i.e.* immobilising metals in the environment to prevent further distribution). These techniques are environment-friendly and can be used for large-scale and diffuse contaminations (Vangronsveld *et al.* 2009; Wu *et al.* 2010; Seth *et al.* 2012). However, to improve the development of efficient strategies, knowledge concerning metal uptake, translocation and detoxification in plants should be extended. During the past 30 years, *Arabidopsis thaliana* is the model organism of choice to unravel molecular responses. Thousands of biologists developed important tools that greatly stimulated plant research with the expanding fields of genetics and molecular biology (Meinke *et al.* 1998; Somerville & Koornneef 2002; Koornneef & Meinke 2010).

1.1.2 *Uptake and transport of cadmium in plants*

As sessile organisms, plants are constantly subjected to environmental stresses that can change their growth conditions and metabolic homeostasis. The similarity between Cd as a divalent cation and essential elements results in accumulation of this non-essential element. In this regard, Cd is taken up into plant cells by Ca, Fe and Zn transporters or channels of low specificity. In addition, the uptake of Cd cations is stimulated by the negative membrane potential and the presence of intracellular Cd binding and sequestration sites (Benavides *et al.* 2005; Clemens 2006). Once Cd passes the cell membrane, it is trapped inside the cell through selective binding with high affinity S-, N- or O-ligands (Clemens 2006; Van Belleghem *et al.* 2007). Subsequently, Cd-ligand complexes can be transported into the vacuole or other organelles. Vacuolar sequestration has been proposed via a Cd^{2+}/H^{+} antiporter CAX2 (Hirschi *et al.* 2000) or transporters of the ATP-binding cassette (ABC) family (Mendoza-Cozatl

et al. 2008). However, a fraction of the vacuolar Cd can be transported back into the cytosol by transporters like Nramp3 (Thomine *et al.* 2003). Next, translocation to the shoots occurs via xylem loading of Cd bound to high-affinity ligands (Gong *et al.* 2003), which has been associated with efflux pumps such as HMA4 (Verret *et al.* 2004).

In order to cope with elevated Cd levels, plants possess defence strategies related to the cellular free Cd content on one hand [e.g. metal exclusion, cell wall binding, chelation and sequestration (Hall 2002)] and the regulation of cellular responses on the other hand [e.g. repair of stress-damaged proteins, antioxidative defence (Hall 2002)]. To limit free metal concentrations, the synthesis of specific chelators and subsequent sequestration of metal complexes are of major importance. Cadmium ions preferentially bind to thiol (-SH) groups, present on cysteine residues of glutathione (GSH), phytochelatins (PCs) and metallothioneins (MTs). These chelators scavenge free Cd ions and prevent them from binding to potentially susceptible thiol groups of essential proteins, and hence disturb their function (Herbette *et al.* 2006; Verbruggen *et al.* 2009a). Phytochelatins are polymerised forms of GSH; their multiple thiol-binding sites render them very efficient in Cd chelation. As mentioned above, vacuolar sequestration of Cd occurs via ABC transporters, preventing them from damaging vital processes in the cell (Mendoza-Cozatl *et al.* 2008). Although MTs have a high cysteine content, they are mainly associated with Cu homeostasis. However, together with GSH and PCs, elevated MT levels have shown to increase both Cu and Cd tolerance, suggesting overlapping functions (Cobbett & Goldsbrough 2002; Guo *et al.* 2008).

1.1.3 *Cadmium induces an oxidative challenge in plants*

Besides Cd chelation, plants developed a well-equipped antioxidative defence system to manage the Cd-imposed oxidative challenge, characterised by elevated production of reactive oxygen species (ROS). Since Cd is not redox-active, it generates ROS indirectly, either via stimulating ROS producing enzymes (e.g. NADPH oxidase, lipoxygenase) or disturbing proteins involved in electron transport chains or antioxidative defence (DalCorso *et al.* 2008; Sharma & Dietz 2009; Keunen *et al.* 2011a). The production of ROS starts with molecular oxygen (O_2) acting as an electron acceptor. Although singlet oxygen ($^1O_2$) is formed by rearrangement of O_2 electron spins upon excitation, the

remaining ROS results from a univalent reduction of O_2: superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). All ROS molecules are strong oxidising compounds and hence potentially harmful for cell integrity. First, lipid peroxidation can cause membrane instability and result in ion leakage (Demidchik *et al.* 2010). Also protein denaturation and DNA damage are associated with Cd-induced ROS (Bertin & Averbeck 2006). Whether the accumulation of ROS will act as a damaging or signalling factor depends on the balance between ROS production and scavenging (Miller *et al.* 2008; Cuypers *et al.* 2012a). Plant's antioxidative defence system consists of both a detoxification and a regeneration part (Fig. 1.1) (Gross *et al.* 2013). In the first part, superoxide dismutase (SOD) converts $O_2^{\bullet-}$ to H_2O_2. This is an enzymatic reaction catalysed by a metal cofactor that resides within SOD. Depending on the cofactor, the following isoforms exist in plants: CuZnSOD, FeSOD and MnSOD (Kliebenstein *et al.* 1998; Alscher *et al.* 2002). The resulting H_2O_2 is primarily neutralised by catalase (CAT) and ascorbate peroxidase (APx). In contrast to APx, the CAT-mediated reaction does not require an additional electron donor, providing a high reaction speed when necessary. The reaction involving APx consists of two central elements in the antioxidative defence system: the GSH and AsA redox couples, formed by the ratio of their reduced (GSH and AsA) and oxidised [glutathione disulfide (GSSG) and dehydroascorbate (DHA)] forms (Mittler *et al.* 2004; Bielen *et al.* 2013). In this cycle, H_2O_2 reduction coincides with oxidation and reduction of these redox couples, as shown in the regeneration part. In this process, AsA acting as a substrate for APx, is oxidised to monodehydroascorbate (MDHA), which can be reduced back via monodehydroascorbate reductase (MDAR). However, MDHA can also spontaneously dismutate to AsA or dehydroascorbate (DHA), which in turn is reduced by dehydroascorbate reductase (DHAR). Whereas MDAR uses NADPH as reducing equivalent, DHAR depends on GSH for the reduction of AsA. The resulting GSSG can be converted back by the NADPH-dependent glutathione reductase (GR), completing the cycle (Mittler *et al.* 2004). Gene expression and activities of enzymes involved in the AsA-GSH cycle are increased upon exposure to metals, suggesting a requirement for increased activity of the cycle under these conditions (Paradiso *et al.* 2008; Drazkiewicz *et al.* 2010). In addition, AsA can also neutralize ROS non-enzymatically and it is involved in the

regeneration of two antioxidants. First, the reduction of *α*-tocopherol depends on AsA. This lipid-soluble vitamin is located in membranes, where it scavenges both ROS and lipid peroxyl radicals (Wang & Quinn 2000). Secondly, AsA is a cofactor for violaxanthin de-epoxidase, an enzyme involved in formation of zeaxanthin in the photoprotective xanthophyll cycle (Shao *et al.* 2008). Also GSH has primary antioxidant capacities and acts as a substrate for the regeneration of other essential antioxidants besides AsA (e.g. glutaredoxins) (Mittler *et al.* 2004; Foyer & Noctor 2005a; Bashandy *et al.* 2010). The regulation of the different roles of GSH and the precise mechanisms by which it acts as a signal transducer under metal-induced oxidative stress is currently under intense investigation and will provide essential information to understand the cellular responses to metal toxicity. Therefore the remainder of this introduction highlights on GSH and its involvement in: (1) metal homeostasis, (2) antioxidative defence, and (3) signalling under metal stress.

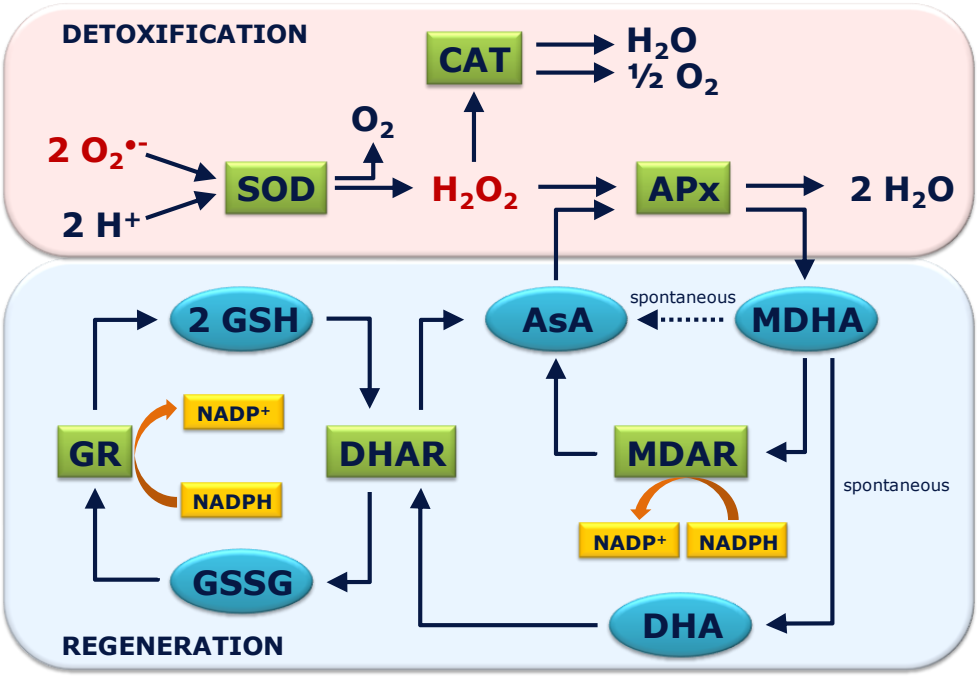

Fig. 1.1 *The antioxidant system in plants (modified from Gross et al. 2013). Abbreviations: ascorbate (AsA), ascorbate peroxidase (APx), catalase (CAT), dehydroascorbate (DHA), dehydroascorbate reductase (DHAR), glutathione (GSH), glutathione disulfide (GSSG), glutathione reductase (GR), hydrogen peroxide (H_2O_2), monodehydroascorbate (MDHA), monodehydroascorbate reductase (MDAR), superoxide ($O_2^{\bullet-}$), superoxide dismutase (SOD).*

1.2. Glutathione biosynthetic pathway and its regulation

Glutathione (γ-glutamyl-cysteinyl-glycine) is a widely distributed tripeptide found at millimolar concentrations (0.5–10 mM) in plant cells (Noctor *et al.* 2012). It is synthesised from three amino acids in two ATP-dependent steps, beginning with the formation of a peptide bond between γ-glutamate and cysteine by γ-glutamylcysteine synthetase (GSH1) and the subsequent addition of glycine, catalysed by glutathione synthetase (GSH2) (Fig. 1.2: GSH biosynthesis) (May *et al.* 1998). Genome sequencing of *Arabidopsis* indicated that both enzymes, GSH1 and GSH2, are encoded by single genes. Mutational knockouts in one of both genes have lethal phenotypes, pointing towards a single pathway for GSH synthesis in plants. Exogenous administration of GSH *in vitro* could partially rescue the development of mutant embryos, demonstrating that endogenous GSH is essential for seed maturation and during germination (Cairns *et al.* 2006; Lim *et al.* 2011). Therefore, in order to investigate the importance of GSH under metal stress, GSH deficient conditions in mutants due to decreased GSH1 activity are preferred instead of knockout plants (Lee *et al.* 2003b). Also the GSH1 inhibitor buthionine sulfoximine (BSO) is often used to alter the pool of cellular GSH. A study in Cu-exposed *Silene cucubalus* demonstrated increased lipid peroxidation and a more oxidised GSSG/GSH ratio after Cu treatment (20 µM). Depletion of GSH by BSO-pre-treatment significantly increased the oxidative damage by Cu (de Vos *et al.* 1992). Recently, Wójcik and Tukiendorf (2011) exposed *Arabidopsis* to Cd, with or without addition of BSO, to investigate GSH adaptation to Cd stress (50 and 100 µM). This experiment showed that treatment with BSO increased Cd toxicity and both GSH content and PC accumulation were more than 96% reduced (Wójcik & Tukiendorf *et al.* 2011). A study investigating the compartmentalisation of GSH biosynthesis shows that GSH1 is restricted to plastids, whereas GSH2 is largely localised in the cytosol and less than 10% is active in plastids (Wachter *et al.* 2005). This observation is consistent with earlier conclusions that export of reduced sulfur from the plastids is mainly in the form of γ-glutamylcysteine (γ-EC), a precursor of GSH (Meyer & Fricker 2002).

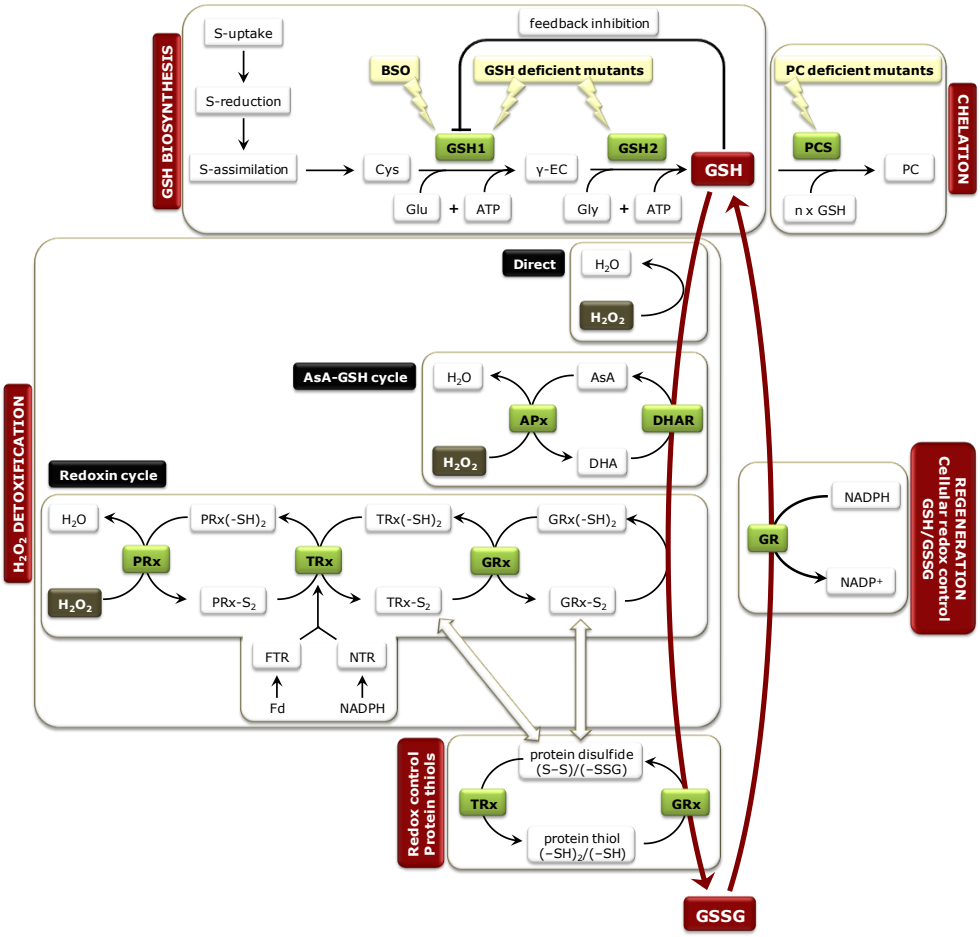

Fig. 1.2 *Overview of GSH biosynthesis and its involvement in chelation and redox control. Glutathione is made of three amino acids: γ-glutamate (Glu), cysteine (Cys) and glycine (Gly) by γ-glutamylcysteine synthetase (GSH1) and GSH synthetase (GSH2) and depends on sulfur (S) availability. Multiple GSH molecules are polymerised by phytochelatin synthase (PCS) to form phytochelatins (PCs). Several antioxidative defence pathways are interconnected with GSH in order to remove excess hydrogen peroxide (H_2O_2). The first defence pathway represents direct non-enzymatic GSH oxidation. Secondly, the ascorbate(AsA)-GSH cycle is displayed in which AsA and GSH are successively oxidised and reduced to allow AsA peroxidase (APx) to neutralize H_2O_2. Thirdly, the two major thiol-redox enzymes glutaredoxin (GRx) and thioredoxin (TRx) are presented that complement the GSH system in redox signalling ether by recycling peroxiredoxin (PRx) that neutralises H_2O_2 or through redox control of protein thiols. In order to investigate the role of GSH in metal-stressed plants, inhibitors of GSH synthesis [i.e. buthionine sulfoximine (BSO)] or deficient mutants are used. Abbreviations: dehydroascorbate (DHA), dehydroascorbate reductase (DHAR), ferredoxin (Fd), ferredoxin-dependent thioredoxin reductase (FTR), γ-glutamylcysteine (γ-EC), NADPH-dependent thioredoxin reductase (NTR).*

The most important factors affecting GSH synthesis rate are sulfur availability and GSH1 activity. Metal toxicity increases both factors in order to meet the elevated GSH demand in cells to ensure detoxification and survival. Sulfur is taken up by plants from the soil as sulfate. After reduction, it is assimilated into bio-organic compounds, with cysteine being the first product (Fig. 1.2: GSH biosynthesis). Generally, this pathway is regulated by demand for reduced sulfur. Since GSH is an important storage form of reduced sulfur in cells, high demands for GSH due to metal stress stimulate sulfate uptake, reduction and assimilation in order to meet the needs of cysteine for GSH and PC biosynthesis (Queval *et al.* 2009; Davidian & Kopriva 2010). This is consistent with studies in maize roots exposed to Cd, Cu and Zn that demonstrate an increased expression of sulfate transporters (e.g. ST1) accompanied with an elevated sulfate uptake (Heiss *et al.* 1999; Nocito *et al.* 2002; Nocito *et al.* 2006). For the reduction of sulfate under Cd stress, increased transcripts of sulfate reductases (e.g. ATPS, APSR) could be linked with alterations in GSH1 (Heiss *et al.* 1999) and specific isoforms of serine acetyltransferase (SAT) and *O*-acetylserine(thiol)lyase (OASTL), the first and last enzyme of cysteine synthesis, respectively. These findings further support the notion for a coordinate transcriptional regulation of sulfur assimilation and GSH synthesis genes as part of the cellular response to Cd exposure (Dominguez-Solis *et al.* 2001; Howarth *et al.* 2003).

The rate-limiting step of GSH biosynthesis is catalysed by GSH1, which is regulated at three levels. First, feedback inhibition of GSH1 activity by GSH has often been considered a fundamental control over GSH synthesis. Alleviation of this feedback inhibition under GSH-consuming conditions is possibly an important mechanism driving accelerated rates of GSH synthesis in response to stress (Hell & Bergmann 1990; Noctor *et al.* 1998a; Noctor *et al.* 2002; Noctor *et al.* 2012). Under metal stress it is well-established that the regulation of GSH biosynthesis undergoes a significant change. Metals increase GSH oxidation and PC production, resulting in a depletion of cellular GSH levels and consequently, the feedback inhibition is released (Fig. 1.2: GSH biosynthesis) (Xiang & Oliver 1998; Zhu *et al.* 1999a). Secondly, *de novo* synthesis of GSH1, but also GSH2, may be enhanced by metal-induced stress (Noctor *et al.* 1998a; Schäfer *et al.* 1998; Xiang & Oliver 1998; Semane *et al.* 2007; Jozefczak *et al.* 2014). The 5'-untranslated region of the *GSH1* gene was shown to interact with a redox-

sensitive repressor-binding protein that was released upon oxidation (Xiang & Oliver 1998; Xiang & Bertrand 2000; Noctor *et al.* 2002; Foyer & Noctor 2005a). Finally, evidence has been presented that GSH1 activity is regulated via post-translational redox controls. Recently, it has been shown that the plant GSH1 enzyme forms a homodimer linked by two redox-sensitive disulfide bonds (S–S). Although the exact mechanistic details are still subject of discussion, reduction of these bonds (–SH) is suggested to be associated with a conformational change and significant inactivation of the enzyme (Hell & Bergmann 1990; May *et al.* 1998; Hothorn *et al.* 2006; Gromes *et al.* 2008). This redox regulation probably contributes to the well-established up-regulation of GSH synthesis in response to oxidative stress conditions like metal toxicity. Exposure of *Arabidopsis* plants to Cd confirms that the distribution of GSH1 is shifted towards the more oxidised and thus active form (Hicks *et al.* 2007). However, it remains unclear whether this mode of regulation is the underlying mechanism of feedback inhibition (Noctor *et al.* 2011).

Two structural features defining GSH are a thiol on cysteine and a *γ*-glutamyl linkage. Since it is the most abundant intracellular thiol as well as *γ*-glutamyl compound, important biological functions are evident for GSH. In general, the physiological significance of GSH in plants may be divided into two categories. First, GSH is an important pool of reduced sulfur and regulates sulfur uptake at the root level. Secondly, GSH contains one cysteine molecule forming the centre of its biological functions in chelation but also in the antioxidative defence and redox control. The SH-group on cysteine is the link through which diverse defence pathways are combined in GSH that will be further discussed in this review. The distinctive *γ*-glutamyl linkage prevents GSH from degradation by common proteases. One way of GSH breakdown is the transfer of *γ*-glutamate to other dipeptides, catalysed by *γ*-glutamyl transpeptidase (Foyer *et al.* 2001; Shaw *et al.* 2005). In another pathway, glycine is removed from GSH by a carboxypeptidase in vacuoles. The remaining dipeptides are metabolised by dipeptidases (Foyer *et al.* 2001). Questions remain about cellular/tissue specificities and activities of degrading enzymes against GSH compared to GSSG or GS-conjugates (Noctor *et al.* 2011). Also the influence of metal stress on GSH breakdown rates is yet to be explored.

1.3. Glutathione and metal homeostasis

About one third of all structurally characterised proteins are metalloproteins, having an essential metal in their active centre. However, the same chemical properties that make metal ions indispensable for biological systems are the reason why they can become toxic when present in excess. A tightly controlled metal homeostasis network is needed to adjust fluctuations in metal availability in order to ensure proper distribution of metals and prevent toxic metal accumulation. Upon entering the cell, two mechanisms are present to bind metals: specific chaperones that deliver essential metals directly to their cellular site of action (Mira *et al.* 2001; Puig *et al.* 2007), and chelators that neutralize and sequester excess free metal ions (Cuypers *et al.* 2009; Verbruggen *et al.* 2009a). Although chaperones normally act under control conditions, metal toxicity will also enhance their expression to prevent free damaging metal ions. Excess Cu ions have been shown to induce *COX17* expression (Balandin & Castresana 2002) and Cd toxicity can increase *CCH* transcripts (Semane *et al.* 2010), two genes encoding Cu chaperone proteins. Chelators like GSH and PCs on the other hand, are major contributors to metal detoxification in plants. Therefore, metal toxicity (e.g. Ag, Cd, Cu, Hg, Zn) increases their concentrations in order to bind metals and subsequently sequester the ligand-metal complexes (Cobbett 2000; Remans *et al.* 2012a; Sobrino-Plata *et al.* 2013; Jozefczak *et al.* 2014).

In general, almost all metals strongly bind to thiol groups of cysteine amino acids, making free cysteine an effective chelator of metal ions. However, when bound to a redox-active metal, cysteine is rapidly oxidised and the reduced metal might undergo a Fenton reaction and form highly toxic hydroxyl radicals ($^{\bullet}OH$; Fig. 1.3). Therefore, keeping free cysteine levels low (up to 50 µM) is necessary to protect against these oxidants. This thiol oxidation by transition metals is greatly reduced after blocking the cysteine amino group by conjugation with glutamate and even further with glycine to form GSH. In this way, a cell can contain a low cysteine concentration and high millimolar GSH concentrations without triggering deleterious Fenton reactions (Fahey & Sundquist 1991). Glutathione protects potentially susceptible cysteine-rich proteins from binding free metal ions and consecutively affecting their function. After forming nontoxic complexes with metals, GSH facilitates their sequestration away from sensitive

sites in cells (Herbette *et al.* 2006; Verbruggen *et al.* 2009a; Foyer & Noctor 2011). Several studies clearly demonstrate the induction of GSH-metal complexes after metal exposure (Cobbett 2000; Cobbett & Goldsbrough 2002; Semane *et al.* 2007; Van Belleghem *et al.* 2007; Verbruggen *et al.* 2009a; Remans *et al.* 2012a; Jozefczak *et al.* 2014). However, not only free metals but also potentially dangerous xenobiotics like herbicides, and metabolites such as anthocyanins, are bound to GSH. The enzyme catalysing these conjugations is glutathione S-transferase (GST) (Marrs 1996; Foyer *et al.* 2001). The activity of GST is shown to be increased in *Arabidopsis* after Cu or Cd treatment in order to stimulate free metal binding (Semane *et al.* 2010; Skorzynska-Polit *et al.* 2010). In plants, the majority of toxic components are translocated and stored into vacuoles (Cobbett & Goldsbrough 2002; Clemens 2006; Klein *et al.* 2006; Yazaki 2006). A tonoplast multidrug resistance-associated protein (MRP) transporter of the ABC type couples ATP hydrolysis to the transport of these GS-conjugates across the vacuolar membrane (Noctor *et al.* 1998a; Verbruggen *et al.* 2009a).

$$2\ O_2^{\bullet -} + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

$$\mathbf{M^{(n)}} + O_2^{\bullet -} \longrightarrow \mathbf{M^{(n-1)}} + O_2$$

$$\text{Fenton}\quad \mathbf{M^{(n-1)}} + H_2O_2 \longrightarrow \mathbf{M^{(n)}} + HO^{\bullet} + OH^-$$

$$\text{Haber Weiss}\quad O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + HO^{\bullet} + OH^-$$

Fig. 1.3 *Fenton and Haber Weiss reaction; oxidised transition metal ($M^{(n)}$), reduced transition metal ($M^{(n-1)}$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$, OH^-).*

Phytochelatins [γ-$(EC)_n$-glycine] are polymerised forms of 2 to 11 GSH molecules, produced by phytochelatin synthase (PCS), a γ-EC transpeptidase (Fig. 1.2: Chelation). Their multiple thiol-binding sites have increased affinity for metals and render PCs more efficient in chelating several metal ions (Noctor *et al.* 1998a; Verbruggen *et al.* 2009a). In leaves of *Arabidopsis* plants exposed to Cd, *PCS1* expression and PC levels (mainly PC2) were strongly induced (Xiang & Oliver 1998; Semane *et al.* 2007). Also after Cd exposure in liquid culture PC levels were induced in a time- and concentration-dependent manner, whereas a less pronounced stimulation was found after Cu exposure (Xiang & Oliver 1998; Semane *et al.* 2007). Metals activate PCS through a metal-specific binding site on the enzyme; removal of the metal ion from PCS inactivates the enzyme again (Vatamaniuk *et al.* 2000; Ogawa *et al.* 2011). Metal chelation through PCs is an

important defence strategy in metal homeostasis as PC-deficient *Arabidopsis* mutants are shown to be hypersensitive to metals like Cd, Hg and As (Howden *et al.* 1995a; Hall 2002). Studies using HPLC data show significant increases in total thiol content after all kinds of metal exposure from different categories, *i.e.* essential *versus* non-essential, redox-active *versus* non-redox-active (Grill *et al.* 1987; Howden *et al.* 1995b; Zhu *et al.* 1999b; Mendoza-Cozatl *et al.* 2008; Carrasco-Gil *et al.* 2011). Although the total thiol concentration is increased, we must distinguish between its four constituents, cysteine, *γ*-EC, GSH and PCs. Cysteine was increased in *Brassica juncea* seedlings after Cd exposure (Zhu *et al.* 1999b), whereas they remained constant in *Brassica napus* plants exposed to Cd (Mendoza-Cozatl *et al.* 2008) and *A. thaliana* plants exposed to Hg (Carrasco-Gil *et al.* 2011). Since free cysteine can be toxic, it is kept at low concentrations in cells through its conversion into peptides. The following constituent in GSH biosynthesis, *γ*-EC, accumulated to a great extent in Cd-exposed plants (Zhu *et al.* 1999b; Mendoza-Cozatl *et al.* 2008), whereas the concentrations of GSH decreased or remained stable upon Cd or Hg exposure (Howden *et al.* 1995b; Semane *et al.* 2010; Carrasco-Gil *et al.* 2011). Whereas GSH1 is the rate-limiting step in GSH biosynthesis under normal conditions, after metal treatment however, it is suggested that GSH2 becomes rate limiting. Consistent with this hypothesis, data confirm a reduced GSH2 activity after metal treatment (Burzynski 1990). This explains why over-expression of *GSH2* does not affect GSH levels in unstressed plants (Foyer *et al.* 1995; Zhu *et al.* 1999a) but under metal stress it may alleviate the depletion of GSH and enhance PC synthesis resulting in an increased metal tolerance (Zhu *et al.* 1999a). Polymerisation of GSH into PCs is absent under control conditions but its synthesis is stimulated in a diverse array of plants upon exposure to a wide range of metals (Grill *et al.* 1987; Jozefczak *et al.* 2014). Furthermore, Grill and co-workers (1987) presented a time course of PC induction and GSH consumption after 200 μM $Cd(NO_3)_2$ to a *Rauvolfia serpentina* cell suspension culture, showing a fast response in which GSH is decreased because it is used as a substrate for PC (Grill *et al.* 1987). This is consistent with a recent *in vivo* study in *A. thaliana* exposed to 5 and 10 μM Cd (Jozefczak *et al.* 2014).

Long-distance translocation of metals from roots to shoots has been proposed to occur amongst others via the xylem in a PC-dependent manner. These findings

suggest that, in addition to the known cellular protection function of PCs, they contribute to maintain a low free metal content in roots. This hypothesis was confirmed by a study of Gong and co-workers in Cd-treated *Arabidopsis* (Gong *et al.* 2003). However, also PCs and Cd were detected in the phloem of Cd-exposed plants, but this retranslocation needs further exploration (Van Belleghem *et al.* 2007; Mendoza-Cozatl *et al.* 2008). Phytochelatins are usually associated with detoxification of non-essential metals. However, not only toxic metals, but also the essential ions like Cu and Zn are bound by PCs suggesting a function for PCs in the regulation of their free cellular concentrations (Hirata *et al.* 2005; Clemens & Persoh 2009). A role for Zn metabolism is clearly demonstrated in PC-deficient *Arabidopsis* mutants (*cad1-3* and *cad1-6*) as deficiency in PCs resulted in a pronounced Zn hypersensitivity and a significant reduction in root Zn accumulation (Tennstedt *et al.* 2009). However, contradictory results indicate that metal-induced PC overproduction might deplete GSH to an extent that causes oxidative stress. Recent studies support this finding as the authors observed an increased PC production, combined with a decrease in GSH content and a more oxidised GSSG/GSH ratio after Cd treatment (1, 5 and 10 µM) in *Arabidopsis*. At the same time, several antioxidative enzymes are activated, indicating increased oxidative stress (Semane *et al.* 2007; Jozefczak *et al.* 2014).

Phytoextraction studies are looking for safe ways to enhance accumulation of excess metals in plants. An interesting course is the overproduction of chelating and binding proteins such as GSH and PC (Pilon-Smits 2005). Studies confirm that over-expression of enzymes in sulfate assimilation, GSH or PC biosynthesis have led to enhanced metal accumulation and tolerance to different metals (Noctor *et al.* 1998a; Rennenberg & Will 2000; Dominguez-Solis *et al.* 2004; Bittsanszky *et al.* 2005). For example, transgenic *B. juncea* over-expressing GSH1 and GSH2 showed enhanced Cd accumulation and was able to significantly reduce Cd and Zn concentrations in metal-contaminated soil due to increased γ-EC, GSH and PCs (Zhu *et al.* 1999a; Zhu *et al.* 1999b; Bennett *et al.* 2003). Over-expression of PCS1 in tobacco plants resulted in enhanced accumulation of Cu, Cd, Pb and Zn in shoots of plants grown on soils polluted with metals (Gisbert *et al.* 2003; Martinez *et al.* 2006). However, when only PCS was over-expressed, it did not always turn out to be beneficial. In *Arabidopsis* and *B.*

juncea, transgenic plants with the highest PCS transcript levels paradoxically were hypersensitive to Cd, whereas plants with moderate over-expression were more resistant (Lee *et al.* 2003b; Gasic & Korban 2007). Additionally, transgenes can behave different in other plant species; *Arabidopsis* AtPCS1 showed high resistance to As and hypersensitivity to Cd in *Arabidopsis* (Li *et al.* 2004), but in *B. juncea* this AtPCS1 improved both As and Cd tolerance (Gasic & Korban 2007). A possible explanation for unexpected or contrasting observations is that forcing the plant to overproduce PCs caused a severe GSH depletion, which disables GSH in fulfilling its other important functions in antioxidative defence and signalling. Also sulfate homeostasis will be disturbed in order to constantly maintain the GSH content. Therefore, metal tolerance is related to the plant's ability to produce PCs and to prevent associated GSH depletion. In accordance, over-expressing genes involved in sulfate assimilation and GSH biosynthesis generally seemed more successful in enabling plants to overcome metal toxicity (Seth *et al.* 2012). Support was found in metal-hyperaccumulating plants that don't seem to rely on PCs, but over-express several antioxidant-related genes and have an enhanced synthesis of GSH to counter the risk of oxidative stress related to high metal uptake (Rascio & Navari-Izzo 2011).

1.4. Glutathione and antioxidative defence

Multiple studies have indicated that plants exposed to any of a diverse array of metals elicit oxidative stress, a process in which the cellular redox balance between pro- and antioxidants is disturbed in favour of the former (Penugonda & Ercal 2004; Cuypers *et al.* 2009; Smeets *et al.* 2009; Cuypers *et al.* 2011; Cuypers *et al.* 2012a; Jozefczak *et al.* 2014). Uncontrolled increases in the steady-state concentrations of these pro-oxidants lead to free radical-mediated chain reactions that target proteins, lipids, polysaccharides and DNA. It has been suggested that metal-induced oxidative stress in cells is partially responsible for the toxic effects of metals (Ercal *et al.* 2001). In order to cope with this oxidative damage, small fluctuations in pro-oxidant concentrations play an important role in signalling processes that regulate cellular responses, resulting in cellular protection and/or acclimation to Cd or excess Cu (Cuypers *et al.* 2012a). Hence, the term "oxidative challenge" is used instead of "oxidative stress"

due to the negative connotation of "stress" (Turrens 2003; Foyer & Noctor 2005a; Cuypers *et al.* 2010; Cuypers *et al.* 2012a; Cuypers *et al.* 2012b).

The primary response of plants to metal stress is the generation of ROS such as $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$, the major contributors to oxidative damage (Gill & Tuteja 2010; Yadav 2010). Depending on the physico-chemical properties of the metal, ROS are formed by different mechanisms. First, even under non-stress conditions, ROS are unavoidable by-products of cellular respiration. Secondly, due to their ability to change in oxidation number, free redox-active metals like Cr, Cu and Fe can directly enhance ROS production through Fenton and Haber Weiss reactions (Fig. 1.3) (Halliwell 2006). Furthermore, metals can alter the cellular redox state indirectly via targeting components of the respiratory chain or antioxidant defence system. Finally, metals can activate pro-oxidative enzymes such as NADPH oxidases and lipoxygenases (Turrens 2003; Mittler *et al.* 2004; Scandalios 2005; Remans *et al.* 2010; Keunen *et al.* 2013b). The last two mechanisms are ways in which also non-redox-active metals (e.g. As, Cd, Co, Hg, Mn, Ni, Pb, Zn) can provoke oxidative stress.

Metal-induced ROS can adversely affect plants at several levels: morphological (e.g. reduced growth, leaf curling), physiological (e.g. photosynthesis, chlorosis, mineral uptake) and biochemical (e.g. membrane leakage, protein inactivation) (Stohs & Bagchi 1995; Maksymiec 1997; Cuypers *et al.* 2000; Benavides *et al.* 2005; Maksymiec & Krupa 2007a; Maksymiec *et al.* 2007b; Cuypers *et al.* 2009; Keunen *et al.* 2011b). Due to their immobility, plants inevitably need to cope with stress conditions. Also the fact that plants both consume and generate O_2 during respiration and photosynthesis is giving them a greater oxidative challenge compared to other eukaryotes. As a result, plant cells respond defensively to oxidative damage by removing ROS and maintaining antioxidant defence compounds at levels that reflect ambient environmental conditions. The antioxidative system contains both enzymatic defences [e.g. SOD, CAT, peroxidase, reductase, redoxin] and metabolites [e.g. GSH, AsA] (Mittler *et al.* 2004; Scandalios 2005; Halliwell 2006; Sharma & Dietz 2009; Bielen *et al.* 2013). Various stress factors, including metals, can disturb the balance between the cellular activities and concentrations of ROS scavengers, leading to cellular damage.

Glutathione is a key player in this antioxidative system, with a significant function in ROS scavenging and as a redox buffer to keep the cellular redox state in balance (Noctor *et al.* 1998a; Schäfer & Buettner 2001; Meyer & Hell 2005). Glutathione exists in reduced (GSH) and oxidised (GSSG) forms. In the reduced state, the thiol group of cysteine is able to donate a reducing electron directly to unstable molecules such as ROS. In donating an electron, GSH itself becomes reactive, but readily reacts with another reactive GSH to form GSSG (Fig. 1.2: Direct H_2O_2 detoxification). In a following step, GSH can be regenerated from GSSG by the action of GR, at the expense of NADPH. Two genes are annotated to encode GR in plants: cytosolic GR1 and GR2, which is dually targeted to plastids and mitochondria. A key characteristic of the cellular GSH pool is its high reduction state due to GR that is constitutively active and inducible upon oxidative stress (Fig. 1.2: Regeneration). In healthy cells more than 90% of the total GSH pool is in its reduced form (Mittler *et al.* 2004). After metal treatment however, GSSG/GSH ratios have been shown to decrease by 65% despite of an increased GR activity (Semane *et al.* 2007; Cuypers *et al.* 2011). This suggests that metal-induced stimulation of GR is insufficient to cope with the massive GSH-consuming effects (direct metal-GSH binding, GSH oxidation, PC synthesis), causing a reduction in free reduced GSH.

In addition to its primary antioxidant capacities, GSH participates in the AsA-GSH cycle that is located in various subcellular compartments (Fig. 1.2: AsA-GSH cycle). This cycle, exclusively existing in plants, is essential for their normal metabolism as well as their defence against oxidative stress. It includes the successive oxidation and reduction of AsA and GSH with a cyclic transfer of reducing equivalents so that the plant-specific APx is able to reduce H_2O_2 to H_2O. The cellular pool of AsA is also maintained in its reduced state by DHAR that uses GSH as an electron donor (Noctor *et al.* 1998a; Mittler *et al.* 2004; Foyer & Noctor 2005a). Highly reduced GSH and AsA pools are essential for an optimal function of the AsA-GSH cycle. Transgenic *Nicotiana tabacum* plants over-expressing GR indicate the critical role of GSH, GR and DHAR in maintaining the AsA pool. Although the GSH pool remained reduced in both plants under oxidative stress conditions, non-transgenic plants displayed a more oxidised AsA pool as compared to transgenic plants. It is essential to keep the AsA pool reduced because APx is rapidly inhibited in the absence of AsA. This study supports the

essential role of GR in the rate of electron supply to DHA (Foyer *et al.* 1991; Noctor & Foyer 1998b). Several studies have demonstrated metal-induced increases in the activities of enzymes involved in this cycle and/or highly oxidised metabolite pools, especially in roots. Therefore, both AsA and GSH act as important redox buffers and their oxidation-reduction ratios reflect the cellular toxicity (Cuypers *et al.* 2000; Smeets *et al.* 2005; Semane *et al.* 2007; Drazkiewicz *et al.* 2010; Cuypers *et al.* 2011). A study of Cuypers and co-workers (2001) illustrate a fast immediate response of the AsA-GSH cycle to Zn toxicity (50 µM Zn) in *Phaseolus vulgaris*. Already 5 hours after Zn exposure, the roots show elevated DHA/AsA and GSSG/GSH ratios due to a decrease in AsA and an increase in GSSG, respectively. The early GSH oxidation might be the cause of AsA regeneration. However, after 5 hours APx activity was decreased due to a lower total AsA concentration, confirming previous findings (Hodges & Forney 2000). This decrease in antioxidant capacity may induce oxidative stress. After 5 days the total AsA content and APx activity were restored in order to deal with the oxidative challenge but the DHA/AsA ratio remained high, indicating that the oxidative challenge was still active. In conclusion, Zn obviously disturbs the GSH balance and hinders the cell from maintaining the AsA pool in the reduced state (Cuypers *et al.* 2001).

In addition to APx, CAT is another major H_2O_2-scavenging enzyme. Compared to APx, it has a low affinity but a high reaction speed for H_2O_2. It has the additional advantage that it is not limited by a substrate. These differences in affinity of APx and CAT suggest that APx is responsible for the fine tuning of ROS concentrations for signalling, whereas CAT might remove the excess ROS as was suggested after metal exposure in *Arabidopsis thaliana* (Cuypers *et al.* 2011). A recent study conducted by Mhamdi and co-workers (2010), provides direct evidence for a role of GR1 in intracellular H_2O_2 metabolism. An *Arabidopsis* GR1 knock-out mutant (*gr1*) and a CAT-deficient mutant (*cat2*) are both characterised by increased GSSG concentrations due to a lack of GR1 on one hand and H_2O_2 accumulation on the other hand. If the major function of GR is to provide GSH for DHA reduction to AsA, down-regulation of GR1 should have a similar response as APx deficiency because APx is inhibited in the absence of reduced AsA. When comparing *gr1cat2* (Mhamdi *et al.* 2010a) and *apx1cat2* (Rizhsky *et al.* 2002) double mutants, an ameliorated phenotype was observed

in *apx1cat2*. Different effects between GR1 and APX1 deficiency are explained by the fact that H_2O_2 is also metabolised through GR-dependent but AsA-independent pathways. Fig. 1.2 displays three different GR-dependent pathways of H_2O_2 metabolism: direct GSH oxidation, the GSH-AsA cycle and the redoxin cycle. The first two pathways have been described above. The redoxin cycle, which is important in cellular (H_2O_2) and protein (-SH) redox homeostasis, is explained in the next section. Although thioredoxin-(TRx-) and GSH-dependent pathways have overlapping functions in plants (Reichheld *et al.* 2007; Marty *et al.* 2009), Mhamdi and co-workers (2010) demonstrated no up-regulation of TRx-dependent genes in increased H_2O_2 conditions like *cat2* and *gr1cat2*, providing evidence that GR1 plays a specific role in intracellular H_2O_2 metabolism. In contrast, GSH-dependent proteins like glutaredoxin (GRx) and GST were transcriptionally up-regulated in these mutants (Mhamdi *et al.* 2010a). Further work is ongoing to confirm the role of GSH status and GR in H_2O_2-trigerred signalling.

1.5. Glutathione redox homeostasis and signalling

Plants constantly face environmental changes as they grow and develop. In order to adapt to their surroundings, plants require both efficient perception and signalling systems. To maintain a reduced state in an otherwise oxidising environment, plants possess internal redox control systems. Elevated ROS production is a general response in plants exposed to metal stress. Although the detrimental effects of ROS cannot be denied, it is the paradoxical concept that the same reactive radicals participate in signal transduction that has become the subject of current research (Foyer & Noctor 2005a; Cuypers *et al.* 2012b). Identification of specialised ROS-generating oxidases in several organisms further supports this concept. For example, NADPH oxidases are suggested to locally create elevated ROS concentrations after metal treatment (Garnier *et al.* 2006; Hao *et al.* 2006; Van Belleghem *et al.* 2007; Heyno *et al.* 2008). *Arabidopsis* seedlings exposed to 5 µM Cd or 2 µM Cu during 24 hours showed metal-specific responses in roots at the transcript level. Cadmium toxicity is associated with an up-regulation of NADPH oxidase, while excess Cu mainly shows a down-regulation of these genes but lipoxygenase genes were induced. These data suggest that metals modulate metal-specific signalling networks in

order to regulate adaptive responses (Remans *et al.* 2010). Generation of secondary messengers like H_2O_2, that are small and able to diffuse over short distances, is a major mechanism to elicit an intracellular signalling response (Fisher 2009). Mitogen-activated protein kinases (MAPKs) are specific ROS sensors that link perception of an environmental signal to downstream targets via sequential phosphorylation of proteins, including transcription factors and enzymes. Several data show that MAPK cascades are involved in signalling activated by different metals in order to translate the information into a biological response (Jonak *et al.* 2004; Yeh *et al.* 2004; Pitzschke & Hirt 2006; Eckardt 2009; Gupta *et al.* 2009; Rao *et al.* 2011; Sinha *et al.* 2011; Opdenakker *et al.* 2012a; Opdenakker *et al.* 2012b). Jonak and co-workers (2004) demonstrated differential activation of several MAPK pathways by Cd and Cu stress in root cells of alfalfa (*Medicago sativa*). These responses occur within half an hour and activate multiple cellular signalling mechanisms, supporting the statement that the cellular response to metal ions and the following signalling pathways are integrated in a signal transduction process (Jonak *et al.* 2004).

1.5.1 Redox control of protein function

Although classical signalling pathways depend on macromolecular interactions, mild oxidants such as H_2O_2 signal through chemical reactions with functional groups of target proteins, resulting in covalent protein modifications at the atomic level. The thiol residue of cysteine, one of the most common amino acids found in proteins, is very useful for structural and regulatory aspects of cells and at the same time a major site of action for ROS. Oxidation of these sulfhydryl groups in proteins results in disulfide bonds that may be required for proper folding to increase enzyme stability or to maintain its activity. Continuous oxidation and reduction of these S–S is possible due to fast and reversible electron transfer between the active site cysteines of thiol-redox enzymes and the cysteines in the target protein (Spadaro *et al.* 2010). Two major thiol-redox enzymes are the NADPH-dependent GRx and TRx (Fig. 1.2: Redoxin cycle). Both systems complement the GSH system in determining protein thiol/disulfide status, a primary factor in redox signalling. In addition, they protect thiol-containing proteins from irreversible oxidation during severe stress conditions (Fig. 1.2: Redox control by protein thiols) (Meyer *et al.* 2008). Although metal-induced TRx and GRx activation has not been studied intensively in plants, there

are indications that GRx is activated by As (Sundaram *et al.* 2008; Lopez-Maury *et al.* 2009). Thioredoxins use two cysteine residues in their active site to reduce protein disulfides. Their active site is reduced in its turn by a ferredoxin- or NADPH dependent thioredoxin reductase (FTR and NTR, respectively). Glutaredoxin on the contrary, can also reduce thiols via reversible glutathionylation of the proteins (*i.e.* the formation of a disulfide bond between GSH and specific cysteine residues) and can be reduced by GSH and NADPH-dependent GR (Ghezzi & Bonetto 2003; Holmgren *et al.* 2005; Rouhier *et al.* 2008; Montrichard *et al.* 2009). Also peroxiredoxin (PRx), which catalyses the reduction of H_2O_2, is recycled by thiols from GSH, GRx and TRx (Meyer 2008; Noctor *et al.* 2012). Only a few studies have investigated the effect of metal toxicity on PRx and show increased transcript levels after treatment with Cd or Cu (Finkemeier *et al.* 2005; Dietz *et al.* 2006; Dietz 2007). Additionally, recent findings using NTR knockouts and GSH deficient mutants identified an alternative reduction of TRx by the GSH-GRx pathway and an NADPH-dependent TRx system as a backup system for GR in *Arabidopsis* (Reichheld *et al.* 2007; Meyer *et al.* 2008; Marty *et al.* 2009; Reichheld *et al.* 2010). In this regard, GSH is essential for the regeneration of the redoxin pool in plants (Bashandy *et al.* 2010). These dithiol-disulfide transitions, including glutathionylation, are the major mechanism in redox control of protein function (Foyer *et al.* 2003; Meyer 2008; Spadaro *et al.* 2010; Noctor *et al.* 2012).

1.5.2 Cellular redox control

Antioxidant redox buffering homeostatically regulates ROS signalling via dithiol-disulfide transitions. In order to maintain the total cellular redox balance, ROS and antioxidants are strongly connected at the level of transcription and translation. Depending on the environmental condition encountered by plants, *i.e.* biotic (bacteria, fungi…) or abiotic (metals, heat…) stress, they may alter the balance between ROS production and removal to enhance or suppress the cellular level of ROS respectively (Foyer & Noctor 2005a).

Cells require a reducing environment that provides the electrochemical gradient needed for electron flow in order to survive. To keep the internal medium in a reduced state, biological evolution created a complex redox buffering system. Many redox couples in a cell [e.g. oxidised/reduced NAD(P), TRx, GRx] work together to provide electron transfer in order to maintain the cellular redox

state. The GSSG/GSH couple is the most abundant in cells and is therefore considered the principal cellular redox buffer. However, the GSH- TRx- and GRx-systems use NADPH as a source of reducing equivalents, demonstrating an interesting thermodynamical connection between these systems (Fig. 1.2: Regeneration). The biological status of a cell is closely linked with its redox environment. The redox state of GSSG/GSH serves as an important indicator of the redox environment, defined by its redox potential ($E_{GSSG/2GSH}$ = −269.55 log([GSH]^2/[GSSG]) mV at 25°C and pH 7.0) and its reducing capacity (total GSH concentration). The redox potential of the cellular environment (E_{hc}) is a measure of the tendency of the GSH redox system to acquire electrons and thereby being reduced. The higher the potential, the more oxidised the GSH redox pool is and therefore the greater its affinity for electrons (Schäfer & Buettner 2001, 2004). The redox environment of a cell changes throughout its life cycle; recent findings suggest that metabolic oxidation regulates the cell cycle and embryonic stem cell differentiation in animals (Menon *et al.* 2003; Yanes *et al.* 2010). Since very similar patterns of GSH recruitment into the nucleus have been observed in plant and animal cells, these mechanisms are possibly the same in all eukaryotes (Markovic *et al.* 2007; Vivancos *et al.* 2010a; Vivancos *et al.* 2010b). At the G1 phase of the cell cycle, GSH is recruited into the nucleus in both plant and animal cells. Cytosolic depletion of GSH causes a readjustment of the intracellular redox environment and oxidative signalling (Vivancos *et al.* 2010a). Immediately, GSH significantly accumulates throughout the cell (Pellny *et al.* 2009), suggesting activation of GSH biosynthesis due to GSH depletion coupled to stromal oxidation. Foyer and Noctor (2011) suggest that posttranslational GSH1 activation and the observed enhanced *GSH2* expression (Vivancos *et al.* 2010b), lead to the increased GSH production and the larger total GSH pool required for redistribution between the daughter cells following mitosis (Vivancos *et al.* 2010a; Foyer & Noctor 2011).

Schäfer and co-workers (2001) developed a theoretical scale of physiological states ranging from cell division over differentiation to cell death, in which $E_{GSSG/2GSH}$ is regarded as a trigger to activate cellular switches between these states. The start of plant development is characterised by proliferating cells with high GSH levels and the most negative redox environment (E_{hc} < −240 mV) (Schäfer & Buettner 2001, 2004). Metal-induced oxidative stress can cause

plants to display slow-growth phenotypes resulting from an increased oxidative load. Evidence was found in CAT deficient *Arabidopsis* mutants with reduced growth that is linked to GSSG accumulation instead of an increased H_2O_2 production (Mhamdi *et al.* 2010c). This GSSG accumulation suggests that these mutants are not capable of maintaining the GSH status at sufficient values to allow the dividing cells to progress rapidly out of the G1 phase (Foyer & Noctor 2011). Glutathione abundance in proliferating cells plays a critical role in development via regulating auxin transport and signalling (Bashandy *et al.* 2010). Studies on metal-induced changes to the cell cycle in plants explain their reduced growth. After treatment with Pb (2.5 mg/L during 30–72 hours), *Allium cepa* L. showed a prolonged cell cycle by 55 to 216% depending on the cell division phase of the cells (Wierzbicka 1999).

More positive redox potentials (E_{hc} from −240 to −200 mV) slow down proliferation and activate nano-switches for differentiation until a maximum is reached and nearly all cells are moved from proliferation to differentiation (Schäfer & Buettner 2001, 2004). The control of the intracellular redistribution of antioxidants, especially GSH, has been suggested to act as a signal in the regulation of the cell cycle (Foyer & Noctor 2011). However, not many studies have been performed in this area on plants exposed to metals. Cells that are not terminally differentiated could proliferate again when an appropriate signal and associated, more negative, redox environment are apparent. Additional increases in E_{hc} can suppress differentiation and when the redox environment cannot be maintained due to stress or damage, death signals are activated and apoptosis is initiated (E_{hc} from −200 to −170 mV). When cells are not capable of activating or responding to these switches, severe oxidative stress will greatly increase the redox potential and necrosis will occur (E_{hc} > −170 mV) (Schäfer & Buettner 2001). From this point of view, the redox environment is a useful tool to determine biological vitality (Dragičević *et al.* 2010). A study shows that in general, plant stress becomes lethal when $E_{GSSG/2GSH}$ exceeds −160 mV due to a signalling cascade initiating apoptosis. Although $E_{GSSG/2GSH}$ is proposed as a universal marker of plant viability and to predict whether seeds may live or die, only few studies are published using this marker (Antunes & Cadenas 2001; Kranner *et al.* 2006). Several studies demonstrate that the cellular redox state is an important characteristic of metal phytotoxicity (Xiang & Oliver 1998; Cuypers

et al. 2002; Cuypers *et al.* 2011). Treatment of *Arabidopsis* with 1 µM Cd during 1 week showed increased GSSG and reduced GSH concentrations, resulting in an elevated GSSG/GSH ratio. These plants seemed able to cope with the metal stress and adopt a new metabolic equilibrium. Plants exposed to 10 µM Cd however, were not capable of maintaining the redox homeostasis and suffered from metal-induced oxidative stress (Semane *et al.* 2007). For both high and low metal concentrations it is interesting to measure $E_{GSSG/2GSH}$ after metal treatment in order to determine cell viability in future studies.

The assessment of the redox environment is straightforward in homogenous fluids. In cells however, compartmentalisation of GSH and GSSG influences the local GSSG/GSH ratio. For example, the endoplasmatic reticulum has a more oxidising environment ($E_{GSSG/2GSH}$ = −180 mV) with respect to the cytosol ($E_{GSSG/2GSH}$ = −232 mV) in order to support and ensure proper protein folding and formation of necessary disulfide bridges (Hwang *et al.* 1992; Schäfer & Buettner 2001). In addition, depending on the total GSH concentration and pH in a cell or organelle, the size of an oxidative event associated with nano-switches will vary. Therefore, findings often seem confusing and contradicting because a certain treatment might change the biological status of one cell, while no response occurs in another. However, one must bear in mind that not all oxidative stimuli create a more oxidised biological state, mild oxidants like H_2O_2 are normal regulators of cellular homeostasis until the capacity to detoxify them is exceeded (Schäfer & Buettner 2001, 2004). Although metal-induced subcellular redistribution of GSH is still in its infancy, the following paragraph summarises the latest findings.

1.5.3 Glutathione compartmentalisation

In order to fully understand the function of GSH in signalling networks, the subcellular compartmentalisation of this key redox player should be further investigated. Immunolocalisation studies with specific GSH-fluorescent labelling revealed that under control conditions GSH is present in all cellular compartments of root and leaf cells, with the exception of the apoplast (Zechmann *et al.* 2008; Zechmann & Müller 2010). At the macroscopic level, GSH is also detected in phloem vessels and vascular parenchyma cells, indicating GSH loading into phloem cells and transport to other plant parts via sieve tubes. Since GSH was not detected in cell walls or intercellular spaces, the

loading is suggested to take place through plasmodesmata (Zechmann & Müller 2010). An oligopeptide transporter (OPT) has been introduced as transporter of GSH, GSSG and even GS-conjugates across the plasma membrane in *B. juncea*, rice and *A. thaliana* (Koh *et al.* 2002; Cagnac *et al.* 2004; Noctor *et al.* 2011). Consistent with its proposed role in long-distance transport, *OPT6* is highly expressed in the vasculature (Cagnac *et al.* 2004). Redundant genes are proposed for OPT6 because knockout mutants display no altered phenotype (Pike *et al.* 2009). As mentioned before, combination of Cd and GSH/PCs in xylem sap of Cd-exposed plants has been observed, indicating long-distance transport of GSH under metal stress (Gong *et al.* 2003; Van Belleghem *et al.* 2007; Mendoza-Cozatl *et al.* 2008).

Glutathione biosynthesis is restricted to the cytosol and plastids, so the newly synthesised GSH must be distributed to other subcellular compartments. Transport of both γ-EC and GSH across the plastid envelope by the chloroquinone-like transporter (CLT) family confirms the finding that GSH1 is exclusively located in the plastid and the major location of GSH2 is the cytosol (Wachter *et al.* 2005; Maughan *et al.* 2010; Noctor *et al.* 2011). Recently, the highest GSH levels were detected in mitochondria, followed by the nucleus (Zechmann *et al.* 2008; Zechmann & Müller 2010). Recruitment of GSH into these compartments suggests the presence of proteins that can increase the permeability of pores for GSH sequestration in the nucleus and mitochondria as is observed for animal cells (Voehringer *et al.* 1998; Noctor *et al.* 2011). Findings of Zechmann and co-workers (2010) that GSH concentrations are the highest in mitochondria, and that even in situations of permanent GSH deficiency, these levels are maintained at the expense of other subcellular GSH pools is interesting for future research. This effect was observed in both plant and mammalian cells using the GSH deficient *Arabidopsis* mutant *pad2-1* (*in vivo*) or the GSH1 inhibitor BSO (*in vitro*) (Green *et al.* 2006; Zechmann *et al.* 2006; Zechmann *et al.* 2008). Since mitochondria cannot synthesize GSH themselves (Wachter *et al.* 2005), this finding demonstrates highly competitive mitochondrial GSH uptake systems. In *Arabidopsis* it is known that mitochondria play a central role in the cellular carbon and nitrogen metabolism. Changes in mitochondrial electron transport and/or mitochondrial ROS production can influence all other organelles (Sweetlove *et al.* 2007; Keunen *et al.* 2011a).

Mitochondria are highly sensitive to redox fluctuations due to Cd toxicity as shown after exposure of *Arabidopsis* cell cultures to 5 µM Cd. In this experiment Schwarzländer and co-workers (2009) demonstrated a redox perturbation in the mitochondria after Cd exposure with important effects on redox signalling. Two possible explanations were proposed. There is either a reduced capacity of the mitochondria to buffer oxidation, or there is a persistent ROS production after treatment due to oxidative damage to the electron transport chain (Schwarzländer *et al.* 2009). Additionally, mitochondria are required to process excess reductants to form a proton gradient across the membrane for respiration (Rhoads *et al.* 2006). In order to prevent massive oxidative damage, mitochondrial $O_2^{\bullet -}$-neutralising MnSOD is accompanied by H_2O_2-scavenging components. The AsA-GSH cycle plays a major role in mitochondria; both enzymes and metabolites of this cycle have been shown to be affected after metal stress (Cuypers *et al.* 2011; Seth *et al.* 2012). Together, this suggests an essential role for mitochondria in both perception and signalling after metal-induced oxidative challenges in plants (Sweetlove *et al.* 2007; Schwarzländer *et al.* 2009; Keunen *et al.* 2011a; Keunen *et al.* 2013a). Mitochondria acting as a sink for GSH may thus play an important role in signalling and H_2O_2-detoxification (Green *et al.* 2006; Zechmann *et al.* 2006) and therefore be an important survival strategy to prevent cytochrome *c*-induced cell death (Fernandez-Checa 2003; Rodriguez-Enriquez *et al.* 2004).

To investigate the influence of Cd on the subcellular GSH compartmentalisation, Kolb and his co-workers (2010) published a study in which *Cucurbita pepo* L. was exposed to 50 µM Cd during 48 hours. Under control conditions, highest GSH-directed immunogold-labelling density was detected in mitochondria, followed by the nuclei as previously described (Zechmann *et al.* 2006; Zechmann *et al.* 2008; Zechmann & Müller 2010). Consistent with previous experiments (Semane *et al.* 2007), all organelles show a strong decrease in GSH content after Cd treatment, reflecting the importance of GSH in Cd detoxification. The absence of labelling in vacuoles demonstrates that GSH-metal complexes, which are not recognised by the present immunohistochemical approach, are sequestered to this compartment after complexation in the cytosol. The well-established decrease in free GSH due to Cd toxicity is thus a consequence of, among others, PC synthesis and metal complexation (Kolb *et al.* 2010; Remans

et al. 2012a; Jozefczak *et al.* 2014). In the study of Kolb and colleagues (2010), a differential Cd-induced compartmentalisation of GSH was found between mesophyll and glandular trichome cells. All organelles from both cell types show a GSH reduction of 30 to 40%, except for the GSH content in nuclei and cytosol of trichome cells, which was decreased between 70 and 76%. These results indicate a possibly crucial role in Cd detoxification for GSH located in these compartments in trichome cells (Kolb *et al.* 2010). Other studies support that glandular trichomes can accumulate and even excrete large amounts of metals and might be considered the major compartment of Cd accumulation in leaves (Isaure *et al.* 2006; Harada & Choi 2008). The cytosol is important for Cd detoxification in these cells since the first contact with metals after uptake into cells occurs here and it is the major compartment for PC synthesis (Kolb *et al.* 2010). A different study using a CAT2-knockout *Arabidopsis* mutant with increased H_2O_2 levels provides evidence that GSSG accumulation in vacuoles and chloroplasts influences the subcellular distribution of GSH as a response to oxidative stress. This GSSG compartmentalisation may play a role in helping to maintain a reduced cytosolic GSH redox status, implying that plant cells are configured to limit large changes in the cytosolic (and possibly nuclear) GSH redox potentials (Tommasini *et al.* 1993; Mhamdi *et al.* 2010c; Noctor *et al.* 2011; Queval *et al.* 2011). Additionally, GSSG sequestration may partially explain why plants can tolerate GSSG increases without inducing cell death (Queval *et al.* 2007a; Mhamdi *et al.* 2010b). Although GSH concentrations in the vacuoles of unstressed plants have long been considered to be low or negligible (Zechmann *et al.* 2008), accumulation of GSSG in this compartment could be a physiologically important part of oxidative stress responses (Queval *et al.* 2011). This vacuolar import suggests MRP transporter activity will be increased in response to stress-induced increases in cytosolic GSSG accumulation and function to reduce such increases. The expression level of several MRP proteins (MRP3, MRP6 and MRP7) is significantly up-regulated in *Arabidopsis* roots after 5 µM Cd exposure. Consistently, two knockout *Arabidopsis* mutants *mrp6*.1 and *mrp6*.2 display more disturbed leaf development when treated with Cd in comparison with the wild-type plants (Gaillard *et al.* 2008). However, the role of GSSG accumulation in the vacuoles and plastids remains to be investigated. Moreover, it remains unclear why equal decreases occur in nuclei (Kolb *et al.*

2010). The exact role of GSH in the nucleus on plant cells is not fully understood. Nevertheless, high ROS and Cd accumulation seem to be unlikely as Cd accumulates mainly in cytosol and vacuoles (Van Belleghem *et al.* 2007). The authors suggest a Cd-induced relocation of GSH from nuclei to the cytosol for complexation. This statement is supported by the large decrease in GSH of more than 50% in both nuclei and cytosol of Cd-exposed trichome cells (Zechmann *et al.* 2006; Van Belleghem *et al.* 2007; Zechmann *et al.* 2008; Kolb *et al.* 2010). A missing link is the detection or labelling of GSSG and GS-conjugates that can be used in future and more detailed experiments in plants under metal stress.

1.5.4 Glutathione signalling

The antioxidant status appears to set the threshold for general plant defence responses. Any stimulus that changes the cellular redox balance may induce the same set of defence-related genes as ROS. Glutathione status in particular has been proposed to be important in relaying oxidative signals originating from ROS. Several studies suggest that changes in GSH status and enhanced ROS pools are equally important in redox signalling (Mou *et al.* 2003; Ball *et al.* 2004; Gomez *et al.* 2004; Noctor *et al.* 2012). Depending on the capacity of the cellular GSH buffer, either a signal is perceived and relayed, resulting in signalling cascades that elicit downstream responses, or signals may be attenuated and even stop (Schäfer & Buettner 2004). However, the exact mechanism of when and how this system relays signals is still under investigation.

Currently, at least two mechanisms have been described by which GSSG concentration or the GSH status can modulate the activity of signalling proteins independently of ROS. Both are posttranslational modifications that modify cysteine residues in catalytic sites or compete with other thiol modifications in order to regulate enzyme activity. First, GSH can change the redox state of thiol groups of proteins that act as redox cofactors. These so-called thiol switches or protein thiol/disulfide exchanges alter either the activity or the redox state of regulatory proteins. Although, elevated protein oxidation is usually associated with oxidative stress, recent findings reveal a fundamental role for this modification in cell signalling. This reversible process is called redox regulation, analogous to the well-described phosphoregulation (Buchanan & Balmer 2005). In plants, TRx is known to regulate several enzymes in this way, however GRx

also couples the GSH redox potential to these changes in thiol/disulfide state (Noctor *et al.* 2012). Although plants contain a large GRx family, much remains to be discovered regarding their functions under normal and environmental stress conditions like metal stress (Lemaire *et al.* 2007). Microbial thiol switches have been intensively reviewed (Paget & Buttner 2003), but for plants only a few studies are conducted. A first study, as mentioned before, demonstrates that GSH1 from *Nicotiana tabacum* forms an active homodimer under oxidising conditions (Gromes *et al.* 2008). Another study hypothesises that an increased GSSG/GSH state serves as secondary messenger connecting the salicylic acid signal induced by a plant systemic acquired resistance with the activity of the non-expressor of pathogenesis-related protein 1 (NPR1). The activity of the latter is increased after reduction of this oligomer into a monomeric form, possibly accomplished by the oxidised GSH pool (Mou *et al.* 2003).

A second mechanism is glutathionylation of regulatory proteins with direct conjugation of GSH to target cysteine residues, another function of GRx (Klatt & Lamas 2000). Increased GSSG or ROS-induced generation of protein thiol radicals both trigger protein glutathionylation. It has been suggested that this process is an important mechanism in sensing and signalling redox disturbances such as increased ROS production (Foyer *et al.* 2003; Meyer 2008). Photorespiration causes *Arabidopsis* plants deficient in H_2O_2-neutralising CAT (*cat2*) to display greatly enhanced GSSG levels that can accumulate up to 90% of the detectable GSH pool, illustrating a close link between H_2O_2 production and changes in GSH status (Noctor *et al.* 2002; Queval *et al.* 2007a; Queval *et al.* 2009; Queval *et al.* 2011). Semane and co-workers (2007) demonstrated enhanced GSSG/GSH levels and GSSG accumulation in *A. thaliana* after 1 week Cd exposure (1 or 10 µM) (Semane *et al.* 2007), resembling the effects of *cat2* suggesting analogous responses. Several hypotheses are proposed that explain this GSSG accumulation. It is suggested that GSSG accumulates because the capacity of GR is insufficient to keep up with H_2O_2-detoxifying enzymes, either because the latter have higher capacities or due to redundancy (APx, CAT, PRx). Others indicate that decreases in NADPH/NADP could influence the redox state of the GSH pool. In conclusion, this limitation of GR activity might be important to allow rapid and sensitive changes in GSSG/GSH in order to fulfil signalling by GSH (Foyer & Noctor 2009; Noctor *et al.* 2012). Glutathionylation and its

significance *in vivo* remains to be revealed in future research work. An interesting example of glutathionylation has been reported by Michelet and co-workers (2005). A specific type of TRx (f-type) has been shown to be glutathionylated on a cysteine residue away from its active site, resulting in a decreased activity towards specific target proteins including NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Michelet *et al.* 2005). Suggestions have been made that GRx is also involved in deglutathionylation (Gao *et al.* 2009). Whether these are important mechanisms of GSH signalling under metal stress needs yet to be revealed.

The first direct evidence that the GSH metabolism and the expression of other components of antioxidative defences are tightly linked was provided in the *Arabidopsis* mutant regulator of APx2 (*rax1-1*). The *rax1-1* mutant is characterised by an enhanced *APX2* gene expression due to a lesion in the gene encoding GSH1 concomitantly with a 50% reduced GSH content. In addition, elevated gene expression of *APX1* and *DHAR1* has also been reported under control conditions in this GSH-deficient *rax1-1* (Ball *et al.* 2004). Cuypers and co-workers (2011) show that Cd (5 and 10 µM) and Cu (2 and 5 µM) stress elicit analogous increased effects on *APX2* expression levels in *Arabidopsis*. Additionally, in the leaves there was an increased *APX1* expression (Cuypers *et al.* 2011). Experiments to further elucidate the effect of GSH deficiency after metal treatment are currently conducted in our laboratory in order to gain a better knowledge on GSH and its multiple roles in plants under metal stress.

1.6. Conclusions

Plants are constantly exposed to environmental challenges; tuning of adaptive responses requires an information cascade that starts with efficient signal perception. For several years, GSH has been considered a central molecule in cellular metabolism and signalling. Many publications are available that demonstrate the complex and integrated regulation of GSH status after environmental triggers, including metal toxicity. This review focuses on three pillars that are interconnected through GSH as a core component in the plant's defence system under metal stress: metal homeostasis, redox homeostasis and detoxification (Fig. 1.4). Not only metals but also other toxic components like xenobiotics are chelated and detoxified by GSH and its derivatives. The cellular

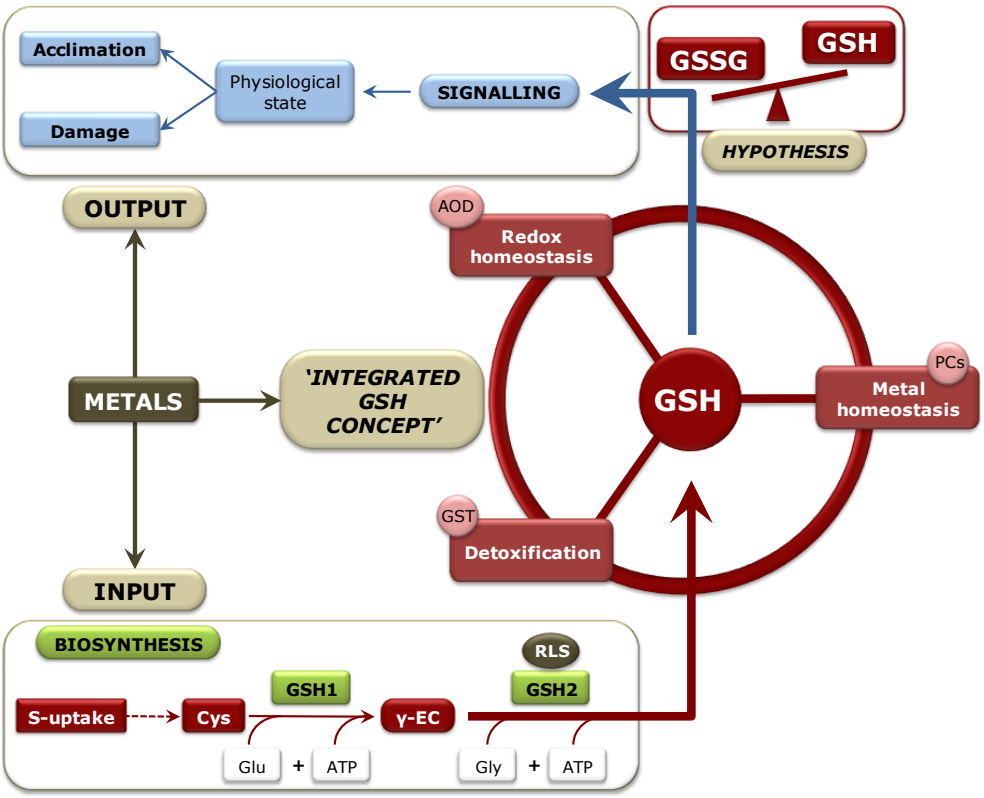

Fig. 1.4 *Schematic overview of the three pillars interconnected through GSH and stimulated under metal stress: metal homeostasis, redox homeostasis and detoxification. The demands imposed by this "integrated GSH concept" under metal stress affect the input into the GSH pool and the output into signal transduction pathways. Metals stimulate the input via an increased GSH biosynthesis including sulfate (S) uptake and assimilation into cysteine. Additionally, metal stress shifts the rate-limiting step (RLS) from GSH biosynthesis from GSH1 to GSH2. There are several indications that the GSH state, including both total GSH content and GSSG/GSH ratio, is involved in these signalling pathways. However, future research is necessary to confirm the role of GSH state in signalling and downstream responses under metal stress. Abbreviations: antioxidative defence (AOD), cysteine (Cys), glutathione S-transferase (GST), glutathione synthetase (GSH2), γ-glutamate (Glu), γ-glutamylcysteine (γ-EC), glycine (Gly), γ-glutamylcysteine synthetase (GSH1) and phytochelatins (PC).*

redox homeostasis comprises the GSH-regulated antioxidative defence. The demands imposed by this "integrated GSH concept" under metal stress affect the input into the GSH pool and the output into signal transduction pathways. Glutathione is directed at the biosynthesis level (total GSH content) and by its cellular redox state (GSSG/GSH), both affected by metals. Upstream signals are sensed by GSH and, depending on the GSH buffering capacity, transmitted into a downstream response. Both dose and duration of the disturbance in the GSH redox state determine these downstream pathways and the outcome of the signalling pathway: damage *versus* acclimation. Glutathione's strategic position between metal scavengers (GSH and PC), detoxification mechanisms (GST) and cellular reductants (AsA-GSH cycle and antioxidative enzymes) makes the GSH redox system (GSSG/GSH) perfectly integrated for signalling functions.

Metal toxicity has been demonstrated to affect GSH at all levels: sulfate uptake and assimilation, biosynthesis of cysteine, GSH and PC and an altered GSSG/GSH balance. Studies using either transformants over-expressing GSH biosynthesis genes or GSH-deficient mutants revealed significant breakthroughs in our understanding of GSH in plant's metabolism. In assessing responses to metal stress, future research should exploit these systems to elucidate the output of the "integrated GSH concept", *i.e.* GSH signalling and downstream responses under metal stress.

Chapter 2

Objectives

The primary focus of this research project is expressed in the following objectives.

Since the industrial revolution, the production, and consequently the emission of both essential and non-essential metals are an integral part of our planet, found in different compartments of the environment. Due to its high toxicity, even low concentrations of the toxic non-essential metal, cadmium (Cd), are associated with mortality (Nawrot *et al.* 2008). Although its concentration in pore waters from control sites is lower than 0.1 µM, anthropogenic activities have led to heavily polluted areas reaching values up to 10 µM in the Campine region in Belgium (Krznaric *et al.* 2009). Despite their non-essential feature, Cd ions are readily taken up by plants via transport systems of essential divalent cations [e.g. zinc (Zn), iron (Fe), calcium (Ca)]. In this regard, as primary producers, plants can quickly accumulate Cd into the food chain (Verbruggen *et al.* 2009a). Exposure to Cd in plants is well known for its disturbances at the following levels: morphology (e.g. growth retardation and leaf chlorosis), physiology (e.g. photosynthesis and membrane damage) and the cellular redox homeostasis [e.g. production of reactive oxygen species (ROS) and oxidation of redox-active molecules]. Plants respond to this Cd-induced challenge by activating their defence mechanisms related to both Cd sequestration and the antioxidative defence (Smeets *et al.* 2009; Cuypers *et al.* 2012a).

Glutathione (GSH) is a central component in defence systems via its redox-active thiol group on cysteine. First, GSH is a major component in Cd chelation and subsequent sequestration due to the high affinity of Cd for thiols, and as a precursor for phytochelatins (PCs). Secondly, GSH is both a primary and a secondary antioxidant, neutralising Cd-induced ROS directly or via GSH-dependent mechanisms like the ascorbate(AsA)-GSH cycle or glutaredoxin (GRx). Finally, the ratio between reduced (GSH) and oxidised (glutathione disulfide, GSSG) forms of GSH represents a major redox buffer, which functions to keep the cellular redox environment reduced (Jozefczak *et al.* 2012). Besides GSH, also AsA is an important antioxidant and redox buffer [AsA-dehydroascorbate (DHA)] due to its reversible oxidation-reduction potential (Noctor 2006). Three characteristics distinguish GSH and AsA from other antioxidants. First, GSH and AsA are coupled to ROS neutralisation by specific enzymes. Secondly, their oxidised forms (GSSG and DHA) are relatively stable. And finally, GSSG and DHA are efficiently recycled by ascorbate reductase (DHAR) and glutathione reductase (GR) (Foyer & Noctor 2011). In this way, a

highly reduced GSH and AsA pool is maintained under control conditions. Changes in their redox state have been associated with redox signalling and subsequent activation of defence mechanisms (Foyer & Noctor 2005b). Both metabolites co-operate with enzymatic antioxidants including the superoxide ($O_2^{\bullet-}$)-scavenging superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2)-neutralising catalase (CAT), ascorbate peroxidase (APx) and redoxins. Together, they work in concert to scavenge ROS and limit uncontrolled oxidation reactions in order to protect plant cells from oxidative damage (Gill & Tuteja 2010).
The main focus of the current study is the role of GSH and AsA as chelator, antioxidant and/or redox buffer in plant responses to sublethal Cd concentrations. The use of *Arabidopsis thaliana* as a model organism led to the opportunity to work with mutants that are deficient in one or both metabolites in order to highlight their importance during the Cd-induced oxidative challenge.

In this regard, the following research questions have been put forward in the outline of this dissertation:

2.1 What is the spatiotemporal involvement of glutathione and ascorbate in cadmium-induced detoxification in *Arabidopsis thaliana* plants?

First of all, a kinetic screening was performed in wild-type *A. thaliana* plants exposed to multiple Cd concentrations and exposure times, providing the basis for more in-depth research in the following chapters. In Chapter 3, fast responses (acute – prolonged) were investigated upon moderate Cd exposure (5 and 10 µM), because recent studies provided evidence that both concentrations result in a highly coordinated defence response after 24 h (Smeets *et al.* 2009; Cuypers *et al.* 2011) and that these concentrations are not lethal during long-term exposure in our set-up (Keunen *et al.* 2011b). The experimental design in Chapter 3 included Cd exposure of three-week-old plants during 0, 2, 24, 48 and 72 h. These time points include one circadian cycle (24 h), short-term (2 h) and a more prolonged exposure (48 and 72 h). In order to gain insight into the sequence of events concerning GSH and AsA upon exposure to Cd in *A. thaliana* roots and leaves, Cd detoxification (*i.e.* chelation and antioxidative defence) was investigated at different levels, including gene expression, enzyme activities and metabolite content.

2.2 What is the effect of glutathione and ascorbate deficiency on cadmium-induced defence mechanisms in *Arabidopsis thaliana* plants?

Glutathione and AsA are abundant, multifunctional antioxidants in plant cells. Both metabolites remove Cd-induced ROS, while GSH also plays a major role in direct Cd scavenging (Jozefczak *et al.* 2012; Bielen *et al.* 2013). Chapter 3 revealed a fast but GSH-depriving PC response in *A. thaliana* roots upon exposure to 5 and 10 µM Cd, while a delayed response was observed in the leaves (Jozefczak *et al.* 2014). The importance of both GSH and AsA in fast Cd detoxification processes was further investigated in Chapter 4 and 5. Therefore, mutants deficient in GSH and/or AsA were exposed to 5 µM Cd during 24 h and 1 or 5 µM Cd during 72 h. Mutations in *A. thaliana* causing severe disturbances in the activity of AsA (*cyt1-2*; Nickle & Meinke 1998) and GSH (*gsh1*; Lim *et al.* 2011) biosynthetic enzymes are lethal. For this reason, mutant plants containing 30-45% wild-type GSH levels (*cad2-1*; Cobbett *et al.* 1998), 40-50% wild-type AsA levels (*vtc1-1*; Conklin *et al.* 1999) or both (*cad2-1 vtc1-1*; Clerkx *et al.* 2004) were used in this study.

Previous studies already reported a higher sensitivity towards Cd for *cad2-1* plants (Cobbett *et al.* 1998). In the current work, it was investigated whether this mutant activates alternative defence mechanisms due to the lack of GSH and PC production. Concerning *vtc1-1* plants, enhanced pathogen resistance has been associated with constitutive priming (Mukherjee *et al.* 2010). In this regard, it was hypothesised that AsA deficiency continuously primes *vtc1-1* mutants and, hence, prepares them to cope with additional stresses like Cd exposure. Genotype- and Cd-specific responses were compared between wild-type and mutant plants concerning both chelation (PC levels) and the antioxidative defence (gene expression, enzyme activities, GSH and AsA redox state and contents).

2.3 How does glutathione regulate alternative antioxidant mechanisms after short-term cadmium-exposure and what is its importance as a substrate for phytochelatins in long-term survival of *Arabidopsis thaliana* plants?

Besides Cd chelation via PCs, it was also revealed in Chapter 3 that SOD is involved in the fast response to Cd-induced oxidative stress in wild-type *A. thaliana* plants (Jozefczak *et al.* 2014). This enzyme converts $O_2^{\bullet-}$ to H_2O_2 and thereby initiates the production of other ROS (Suzuki *et al.* 2012). In addition, it has been shown that *A. thaliana* plants exposed to Cd revealed a transcriptional SOD response in wild-type plants associated with the activation of the squamosa promoter-binding protein-like 7 (SPL7) transcription factor. The latter up-regulates the FeSOD1 (*FSD1*) gene and microRNA398 (*MIR398*), which in its turn represses two CuZnSOD transcripts (*CSD1* and *CSD2*) (Cuypers *et al.* 2011; Gayomba *et al.* 2013). In order to gain more knowledge on the role of GSH in *CSD* expression upon Cd exposure, a more profound study was performed in Chapter 6, including multiple GSH deficient mutants (*cad2-1*, Cobbet *et al.* 1998; *pad2-1*, Parisy *et al.* 2007; and *rax1-1*, Ball *et al.* 2004) and exposure times (24 and 72 h of 5 µM Cd). This GSH dependent *CSD* regulation was investigated at transcriptional, posttranscriptional and enzyme level in order to shed more light on the underlying mechanism.

Although a fast but GSH-depriving PC response has been reported in Cd-exposed wild-type plants (Chapter 3, Jozefczak *et al.* 2014), information on the importance of this PC-mediated response during long-term Cd exposure is contradicting (de Knecht *et al.* 1995; Schat *et al.* 2002). Therefore, in Chapter 7, the accumulation of Cd and PCs in *A. thaliana* wild-type plants was followed during three weeks after exposure to 5 and 10 µM Cd. In order to examine the necessity of Cd-induced PC production for plant survival, also GSH deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) were included.

Chapter 3

Differential response of *Arabidopsis* leaves and roots to cadmium: glutathione-related chelating capacity *vs* antioxidant capacity

Research article – published

Differential response of *Arabidopsis* leaves and roots to cadmium: glutathione-related chelating capacity *vs* antioxidant capacity

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3.1 Abstract

This study aims to uncover the spatiotemporal involvement of glutathione (GSH) in two major mechanisms of cadmium (Cd)-induced detoxification (*i.e.* chelation and antioxidative defence). A kinetic study was conducted on hydroponically grown *Arabidopsis thaliana* (L. Heyhn) to gain insight into the early events after exposure to Cd. Cadmium detoxification was investigated at different levels, including gene transcripts, enzyme activities and metabolite content. Data indicate a time-dependent response both within roots and between plant organs. Early on in roots, GSH was preferentially allocated to phytochelatin (PC) synthesis destined for Cd chelation. This led to decreased GSH levels, without alternative pathways activated to complement GSH's antioxidative functions. After one day however, multiple antioxidative pathways increased including superoxide dismutase (SOD), ascorbate (AsA) and catalase (CAT) to ensure efficient neutralisation of Cd-induced reactive oxygen species (ROS). As a consequence of Cd retention and detoxification in roots, a delayed response occurred in leaves. Together with high leaf thiol contents and possibly signalling responses from the roots, the leaves were protected, allowing them sufficient time to activate their defence mechanisms.

3.2 Introduction

Anthropogenic contamination of soils by cadmium (Cd) is a worldwide problem. Although Cd is a nonessential element, it can enter plant tissues and possibly replace essential elements, rendering the biomolecules they are associated with inactive. Due to its high affinity for sulfhydryl groups, Cd also targets thiol-groups in proteins, thereby changing their redox state and potentially interfering with regulation (Cuypers *et al.* 2009). Plants exposed to Cd show decreased fresh weight and cellular damage generated by Cd directly or by reactive oxygen species (ROS) as a result of Cd-induced oxidative stress (Sharma & Dietz 2009; Cuypers *et al.* 2011). Continued research is needed in the area of Cd toxicity in plants to expand our knowledge and improve strategies for dealing with Cd-contaminated soils (Chary *et al.* 2008; Seth *et al.* 2012).

Chelation and subsequent sequestration of Cd to the least sensitive locations in the cell (e.g. vacuoles) is known to be essential in Cd detoxification (Cobbett &

Goldsbrough 2002). Glutathione (GSH) is a key compound in Cd scavenging due to the high affinity of Cd for the thiol group of the GSH cysteine, and as a precursor for phytochelatins (PCs) (Rauser 2001). It is synthesised in two steps: first a peptide bond is formed between *γ*-glutamate and cysteine by *γ*-glutamylcysteine synthetase (GSH1) to produce *γ*-glutamylcysteine (*γ*-EC); subsequently, glycine is added by glutathione synthetase (GSH2) to produce GSH (Grill *et al.* 1985; May *et al.* 1998). Several studies have shown that Cd toxicity increases sulfur uptake, assimilation of sulfur into cysteine and GSH1 activity, all rate-limiting factors leading to GSH biosynthesis (Howarth *et al.* 2003; Nocito *et al.* 2006). Phytochelatins, which have a higher affinity for Cd, are formed by the polymerisation of 2 to 11 *γ*-EC moieties via phytochelatin synthase (PCS). Several studies confirm that in plants both GSH and PC synthesis increase after exposure to Cd. Apart from PCs, also metallothioneins (MTs), cysteine-rich proteins, are important scavengers of Cd (Cobbett & Goldsbrough; Mendoza-Cozatl *et al.* 2008).

When chelation is insufficient, free Cd ions can cause oxidative damage by disturbing the balance between pro-oxidants (e.g. ROS) and antioxidants in favour of the former. Plants possess several antioxidant mechanisms to cope with ROS, including enzymes such as superoxide dismutases (SOD) and catalases (CAT), as well as metabolites such as GSH and ascorbate (AsA). Glutathione has primary and secondary antioxidative functions (Xiang *et al.* 2001). First, GSH can directly reduce hydrogen peroxide (H_2O_2), resulting in glutathione disulfide (GSSG), which can be regenerated by glutathione reductase (GR), using NADPH as an electron donor. Secondly, in the plant-specific AsA-GSH cycle, H_2O_2 is neutralised by ascorbate peroxidase (APx) via the successive oxidation and reduction of AsA and GSH (Groden & Beck 1979). Large pools of the reduced forms of both metabolites are essential for an optimal antioxidant capacity. Cadmium stress, however, is known to result in the oxidation of these pools and alter the activity of the enzymes involved (Semane *et al.* 2007; Cuypers *et al.* 2011). Additionally, the concentration and redox state of GSH are suggested to be important in transducing oxidative signals originating from ROS in order to activate the antioxidative response (Schäfer & Buettner 2001; Ball *et al.* 2004).

The multiple functions of GSH as Cd chelator, antioxidant and signalling molecule are currently under intense investigation and will provide essential information to better understand the cellular responses to Cd toxicity in plants. The current study focuses on GSH and the sequence of its actions after exposure to Cd.

3.3 Materials and methods

3.3.1 Plant material, cadmium exposure and harvest

Arabidopsis thaliana (L. Heyhn, Columbia background) seeds were surface-sterilised for one minute in 0.1% sodium hypochlorite solution and rinsed thoroughly with distilled water. After three days incubation at 4°C in the dark, seeds were grown in hydroponics under a 12 h/22°C day 12 h/18°C night regime with 65% relative humidity as previously described (Keunen *et al.* 2011b). Light was provided by a combination of blue, red and far-red Philips Green-Power LED modules, simulating the photosynthetically active radiation (PAR) of sunlight. The PAR level reached 170 µmol m^{-2} s^{-1} at the rosette level. A modified Hoagland solution containing macronutrients [505 µM KNO_3, 150 µM $Ca(NO_3)_2.4H_2O$, 100 µM $NH_4H_2PO_4$ and 100 µM $MgSO_4.7H_2O$], micronutrients (4.63 µM H_3BO_3, 0.91 µM $MnCl_2.4H_2O$, 0.03 µM $CuSO_4.5H_2O$, 0.06 µM $H_2MoO_4.H_2O$ and 0.08 µM $ZnSO_4.7H_2O$, 1.64 µM $FeSO_4.7H_2O$) and 0.81 µM Na_2-EDTA was used. After one week of germination, the nutrient solution was continuously aerated and refreshed every three to four days. Three-week-old plants were exposed to 0 (control), 5 or 10 µM $CdSO_4$ administered in the nutrient solution. Root and leaf samples were collected at different time points after start of the exposure (0, 2, 24, 48 and 72 h). Root samples that were harvested for Cd determination were washed for 15 min with 10 mM $Pb(NO_3)_2$ at 4°C to remove surface-bound metals, then rinsed in distilled water. Leaves were only rinsed in distilled water before they were dried at 60°C. Cadmium concentrations were determined by inductively coupled plasma-atomic emission spectrometry (Perkin Elmer, Optima 3000 DV, Bedford, USA) after digestion of dried root and leaf samples in 70-71% HNO_3 using a heat block. The remaining samples were snap frozen in liquid nitrogen immediately after harvest and stored at -80°C prior to further analysis.

3.3.2 Metabolite determination

The concentration of thiobarbituric acid-reactive metabolites (TBArm) was determined spectrophotometrically (UV-Visible Spectrophotometer, UV-1602, Shimadzu Corporation, Japan) as previously described (Cuypers *et al.* 2011). In brief, plant tissue (100 mg) was homogenised with 1 ml 0.1% trichloroacetic acid and centrifuged (10 min, 13200 rpm, 4°C). The supernatant was diluted 3.5 times in 0.5% thiobarbituric acid and heated for 30 min at 95°C. After centrifugation, the absorbance of the supernatant was measured at 532 nm and corrected for unspecific absorbance at 600 nm.

Non-protein thiols were extracted and analysed after derivatisation with monobromobimane by reverse-phase HPLC according to Huguet *et al.* (2012). Briefly, 25 mg aliquots of material ground in 0.1% trifluoroacetic acid, 6.3 mM DTPA (pH ≤ 1) and 10 µM N-acetyl-l-cysteine (internal standard) were filtered and incubated with 280 µM monobromobimane, 125 mM HEPPS (pH 8.2) and 4 mM DTPA (30 min, 45°C, in dark). Thiols were separated on a Nova-Pak C_{18} analytical column (Waters, Milford, USA) at 37°C and eluted with a slightly concave gradient of water and methanol. Fluorescent compounds were identified with Ellman's reagent using a Waters 474 fluorescence detector. Quantification was based on N-acetyl-l-cysteine and GSH standards (0-20 µM) and corrected for derivatisation efficiency (Huguet *et al.* 2012).

Oxidised and reduced forms of GSH and AsA were extracted and analysed by enzymatic assays on a plate reader based on the protocol previously described by Queval and Noctor (2007b). Briefly, samples (100 mg) were ground in 200 mM HCl and adjusted to pH 4.5. Ascorbate was determined spectrophotometrically at 256 nm after addition of 1 U ml^{-1} ascorbate oxidase (from *Curcubita sp.*, Sigma, St. Louis, MO, USA) and corrected for background signal. To measure total AsA, aliquots were incubated with 1 mM DTT (15 min, 20°C) and adjusted to pH 5.6. Total GSH was measured by a kinetic enzymatic recycling assay based on the GSH-dependent reduction of Ellman's reagent (600 µM) and subsequent reduction of GSSG by GR (1 U ml^{-1}) in the presence of NADPH (500 µM). The rate of absorbance change over 5 min is proportional to the GSH concentration which was calculated using a GSH standard curve. In order to measure GSSG, extracts were first incubated with 1% 2-vinylpyridine and centrifuged twice (Queval & Noctor 2007b).

3.3.3 Gene expression analysis

Samples were disrupted under frozen conditions using two stainless steel beads and the Retch Mixer Mill MM 2000 (Retsch, Germany). Isolation of RNA was performed using the *mir*Vana miRNA Isolation Kit (Ambion, Lennik, Belgium). Genomic DNA was removed using the TURBO DNA-free Kit (Ambion) and reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Ambion) with random hexamer priming and equal amounts of RNA input (1 µg). A tenfold dilution of the cDNA was made using 1/10 diluted TE-buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20°C. Quantitative PCR (qPCR) was performed with an ABI Prism 7900HT using Fast SYBR Green chemistry according to the manufacturer's instructions (Applied Biosystems, Paisley, UK). Gene expression was calculated relatively as $2^{-\Delta Cq}$ and was normalised with a normalisation factor based on the expression of reference genes *AT3G18780*, *AT5G55840* and *AT4G34270* for roots and *AT2G28390*, *AT5G25760* and *AT4G34270* for leaves (Remans *et al.* 2008). Gene-specific primers (300 nM unless stated otherwise, Table A.3.1 in Appendices) were designed and optimised using Primer Express (Applied Biosystems).

For the mature microRNA analysis, a final purification of RNA, enriched for small RNAs, was performed (*mir*Vana miRNA Isolation Kit, Ambion). Twenty ng microRNA was primed with three specific primers (*MIR172a*, *MIR398a* and *MIR398b/c*) according to the manufacturer's instructions (Multiplex RT for TaqMan MicroRNA Assay, Applied Biosystems). Copy DNA was diluted tenfold in RNase-free water and stored at -20°C. Gene expression was calculated relatively as $2^{-\Delta Cq}$ and *MIR172a* was used for normalisation. Expression was based on TaqMan Universal PCR (Applied Biosystems) and investigated using 9600 Emulation Mode (ABI Prism 7900HT, Applied Biosystems), according to the manufacturer's instruction. All primers for microRNAs (62.5 nM, Table A.3.1 in Appendices) were purchased from Applied Biosystems. Table A.3.2 in Appendices shows the Reverse Transcription quantitative (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines (Bustin *et al.* 2009).

3.3.4 Native gel electrophoresis

Extracts for enzyme activities were prepared on ice in 1 ml extraction buffer (100 mM Tris, 1 mM EDTA, 1 mM DTT, pH 7.8) and 5 mg of polyvinylpyrrolidone

per 500 mg fresh weight. Protein concentrations were determined (Bradford Protein Assay; BioRad, California, USA) and adequate loading quantities were determined by denaturing polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). Respectively 10 and 30 µg of root and leaf extracts were loaded on gels for non-denaturing PAGE. After separation of protein extracts at 4°C in 10% polyacrylamide gels, SOD activities were determined in gel (Sobrino-Plata *et al.* 2009). After incubating the gels in the dark with 245 µM nitrotetrazolium blue, superoxide ($O_2^{\bullet -}$) was produced in the photo-oxidation reaction of 22 mM tetramethylethylenediamine in the presence of 66 µM riboflavin in 50 mM potassium phosphate buffer with 1 mM EDTA (pH 7.8). Isoforms of SOD were differentiated by incubation (30 min, before staining) with potassium cyanide, which selectively inhibits the activity of the copper/zinc isoform (CuZnSOD), and H_2O_2, which inhibits activity of both CuZnSOD and the iron isoform (FeSOD).

3.3.5 Statistical analysis

Datasets were analysed using linear mixed-effects ANOVA models (Verbeke & Molenberghs 2000). Normal distribution was tested using Shapiro-Wilk, Kolmogorov-Smirnov, Craner-von Mises and Anderson-Darling tests. Transformations (log, square root, inverse, exponential) were applied when necessary to approximate normality. Tukey post-hoc adjustment was used to correct for multiple comparison. All statistical analyses were performed using SAS 9.2 (SAS Institute Inc., version 4.3, Cary, USA). Data are mean values ± standard error (SE) and significance was set at the 5% level ($P < 0.05$).

3.4 Results

Hydroponic cultures of three-week-old *A. thaliana* seedlings were exposed to sublethal Cd concentrations (0, 5 or 10 µM; Keunen et al. 2011) that were detected in soil pore waters in polluted areas (Krznaric *et al.* 2009). The experimental design included exposure during 0, 2, 24, 48 and 72 h. These time points were chosen because they include one circadian cycle (24 h), short-term (2 h) and a prolonged exposure (48 and 72 h).

Generally, Cd concentrations were higher in roots than in leaves (Fig. 3.1). Starting from 2 h of Cd exposure, a time- and dose-dependent increase of root Cd concentrations was observed ($P < 0.0001$), while leaf Cd levels increased

independent of dose and were already at a plateau at 24 h. As a result, the translocation factor, representing the relative translocation of Cd from root to leaf, was lowest in the plants exposed to the highest dose. Compared to non-exposed seedlings, decreases in fresh weight were observed with increasing Cd concentrations after 24 h for the roots and after 48 h for the leaves (Fig. 3.2). Another effect of high Cd exposure was increased lipid peroxidation, which was determined as TBArm. Significant increases were detected after exposure to 10 µM Cd in roots and leaves after 72 and 48 h, respectively (Fig. 3.3).

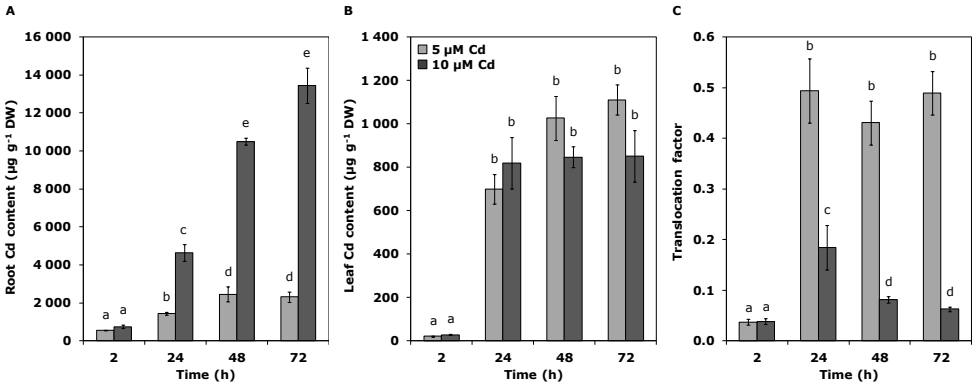


Fig. 3.1 *(A) Root and (B) leaf Cd contents (µg g^{-1} DW) and (C) the corresponding translocation factor from roots to shoots of Arabidopsis thaliana exposed to 5 (□) or 10 (■) µM $CdSO_4$ for different durations (2, 24, 48, 72 h). Note the different scales on the y-axes between both organs. Statistical significance is expressed using lower case letters; n = 3 (two-way ANOVA, P < 0.05).*

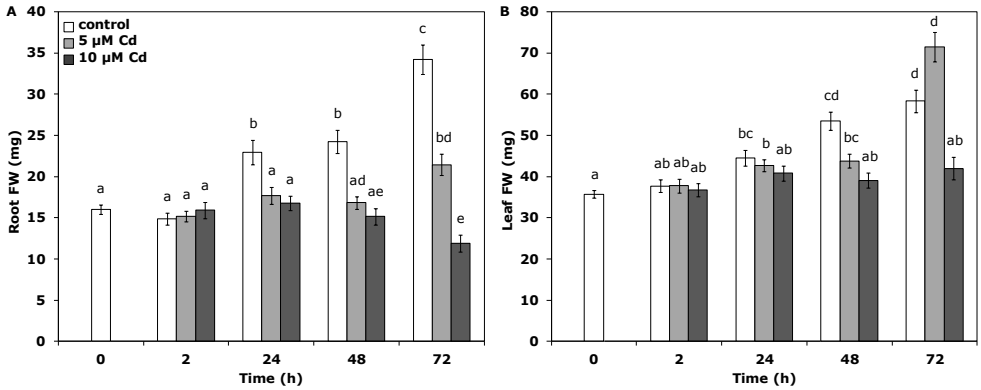


Fig. 3.2 *Weight (mg FW) of roots (A) and leaves (B) of Arabidopsis thaliana exposed to 0 (□), 5 (▒) or 10 (■) µM $CdSO_4$ for different time periods (0, 2, 24, 48, 72 h). Statistical significance is expressed using lower case letters; n = 40 (two-way ANOVA, P < 0.05).*

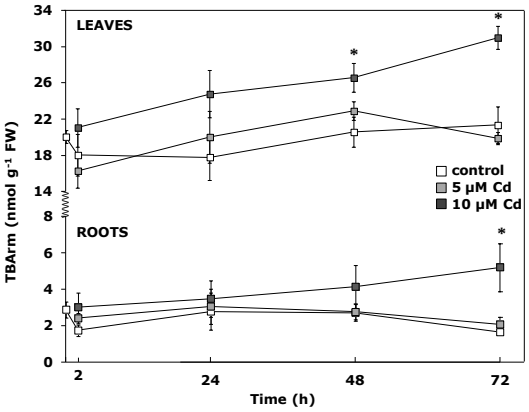
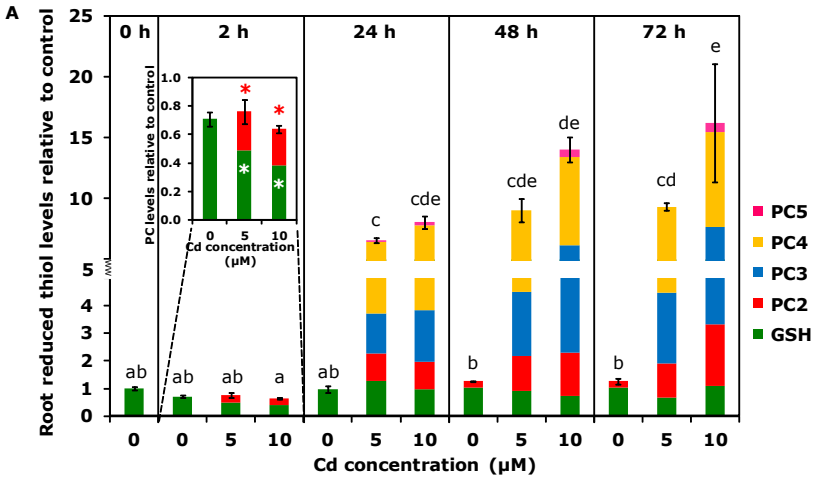


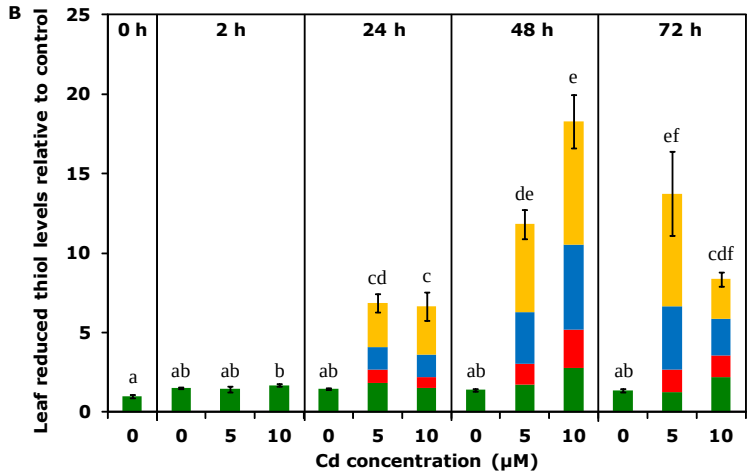
Fig. 3.3 *Thiobarbituric acid-reactive metabolites (TBArm) (nmol g^{-1} FW) in leaves and roots of Arabidopsis thaliana exposed to 0 (□), 5 (▒) or 10 (■) µM $CdSO_4$ for different time periods (0, 2, 24, 48, 72 h); n = 4 (***** significant differences with the correspondding control, one-way ANOVA, P < 0.05).*

3.4.1 Cadmium rapidly activates the thiol metabolism in A. thaliana

Phytochelatins and MTs are well-characterised metal-binding ligands in plant cells. This study reports Cd-induced increases in gene expression levels of *PCS1* (Fig. 3.4) and *MT2a* (Table A.3.3 in Appendices) after 24 h in both organs. Changes in expression of *PCS2* and other MT transcripts were absent or limited. In contrast to *MT2a*, transcript levels of *PCS1* were only transiently enhanced. Since PCS is post-transcriptionally activated at the enzyme level by metal binding (Ogawa *et al.* 2011), thiol profiles were also analysed in order to investigate the actual PC production (Fig. 3.4). Roots exposed to Cd display a fast increase in PC levels. More specifically, after 2 h, the production of PC2 coincided with decreased GSH levels and a constant thiol concentration (see the inset in Fig. 3.4A). After 24 h, the total thiol levels were increased in a dose-dependent manner in both organs ($P < 0.0001$). In leaves however, exposure to 10 µM Cd resulted in decreased PC levels after 72 h compared to 48 h ($P < 0.001$, Fig. 3.4B).



relative expression		2 h	24 h	48 h	72 h
PCS1	5 µM Cd	1.21 ± 0.19	1.74 ± 0.14	1.31 ± 0.28	0.92 ± 0.18
	10 µM Cd	0.97 ± 0.35	1.37 ± 0.16	0.87 ± 0.01	0.72 ± 0.05
PCS2	5 µM Cd	0.82 ± 0.07	0.89 ± 0.28	1.58 ± 0.22	0.99 ± 0.30
	10 µM Cd	0.66 ± 0.20	0.46 ± 0.158	0.99 ± 0.36	0.85 ± 0.16



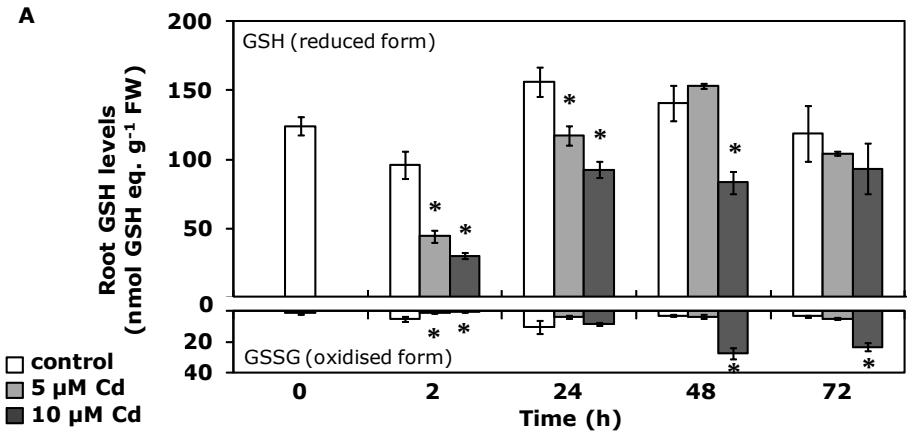
relative expression		2 h	24 h	48 h	72 h
PCS1	5 µM Cd	0.91 ± 0.09	6.43 ± 0.86	1.27 ± 0.09	1.28 ± 0.10
	10 µM Cd	1.15 ± 0.13	4.07 ± 0.64	0.99 ± 0.16	1.03 ± 0.13
PCS2	5 µM Cd	0.73 ± 0.13	2.88 ± 1.11	2.22 ± 0.48	1.26 ± 0.12
	10 µM Cd	0.67 ± 0.06	1.09 ± 0.339	0.97 ± 0.27	0.68 ± 0.19

Fig. 3.4 *Profiling the total glutathione (GSH, ■) and phytochelatins (PC2, ■; PC3, ■; PC4, ■; PC5, ■) related reduced thiol content in Arabidopsis thaliana roots (A) and leaves (B) exposed to 0, 5 or 10 µM $CdSO_4$ for different time periods (0, 2, 24, 48, 72 h). Thiol content is expressed relative to the control at 0 h. Statistical significance in total thiol levels is expressed using lower case letters; n = 4 (two-way ANOVA, P < 0.05). The inset in (A) highlights the data after 2 h in roots in order to demonstrate the significant differences in GSH (■) and PC2 (*) relative to control conditions; n = 4 (one-way ANOVA, P < 0.05). Phytochelatin synthase (PCS1 and PCS2) is expressed relative to the corresponding control in the table below; n = 4 (significant up-regulation, ■; one-way ANOVA, P < 0.05).*

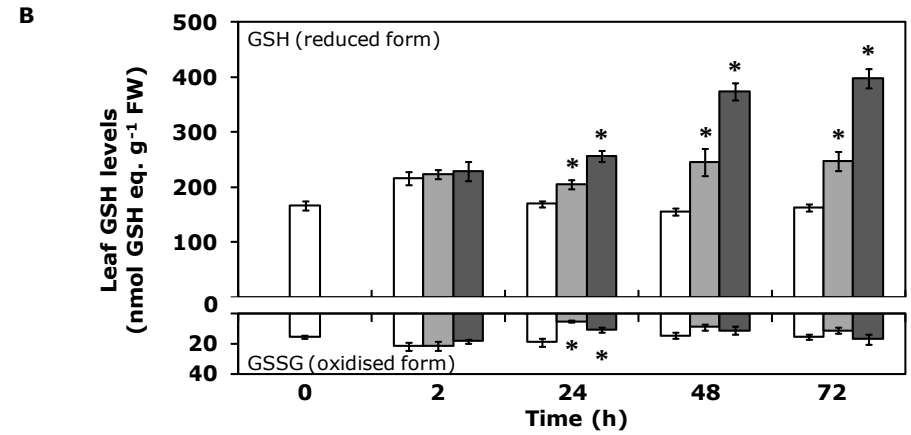
To study the thiol profiles in more detail, the concentration and redox state of GSH were investigated (Fig. 3.5). This metabolite assay confirmed the decrease in root GSH levels after 2 h as stated above. This decline however, was still present 24 and 48 h after the start of exposure to 5 and 10 µM Cd, respectively. After 2 h of Cd exposure, GSSG levels were also reduced but returned to control levels after 24 h. Total GSH levels were significantly diminished after 2 and 24 h. At later time points, GSSG (48 h) and *GR1* transcript levels (72 h) were significantly increased and the redox balance of GSH was shifted towards a more oxidised state ($P < 0.05$) in roots exposed to 10 µM Cd. In contrast to the roots, reduced and total GSH levels in the leaves were not different from the control levels after 2 h of Cd exposure and subsequently increased in a time- and dose-dependent manner ($P < 0.0001$). In combination with decreased GSSG levels at 24 h and a dose-dependent induction of *GR1* expression, a more reduced GSH redox state was apparent ($P < 0.05$). Expression levels of *GR2*, however, did not show significant differences in roots or leaves (data not shown). After 48 h, GSSG returned to control levels and the GSH:GSSG ratio remained favourable to the reduced form of GSH. Additionally, the expression of both GSH biosynthesis genes (*GSH1* and *GSH2*) was increased in both organs after 24 h of exposure to the highest Cd concentration.

3.4.2 MicroRNA-directed regulation of superoxide dismutase during cadmium toxicity in Arabidopsis thaliana

The microRNA398 family of *A. thaliana* is encoded by two members with three loci: *MIR398a*, *MIR398b* and *MIR398c*. Both *MIR398b* and *MIR398c* are identical in sequence and thus undistinguishable at mature transcript levels (Zhu *et al.* 2011). Gene expression analysis (Table 3.1) shows that *MIR398a* transcript levels augmented in roots after 2 h and in leaves after 24 h of Cd exposure. Both *FSD1* and *MIR398b/c* were increased after 24 and 48 h in roots and leaves, respectively. Increased levels of *MIR* were accompanied by a general decrease in both *CSD1* and *CSD2* transcripts. Additionally, three Cu-chaperones were investigated at the transcriptional level: *CCS* was down-regulated after 24 h in both plant organs; *CCH* levels were increased after 24 h in roots and after 48 h in leaves; and *ATX1* was up-regulated but only in leaves after 24 h of Cd exposure.



relative expression		2 h	24 h	48 h	72 h
GR1	5 µM Cd	0.79 ± 0.15	0.88 ± 0.06	0.80 ± 0.18	0.67 ± 0.12
	10 µM Cd	0.63 ± 0.15	1.24 ± 0.05	1.28 ± 0.10	1.32 ± 0.01
GSH1	5 µM Cd	1.17 ± 0.17	0.92 ± 0.24	1.30 ± 0.32	1.02 ± 0.27
	10 µM Cd	1.04 ± 0.33	2.95 ± 0.55	3.51 ± 0.47	2.33 ± 0.26
GSH2	5 µM Cd	1.02 ± 0.10	1.72 ± 0.21	1.49 ± 0.17	1.13 ± 0.24
	10 µM Cd	0.85 ± 0.26	3.86 ± 0.65	3.23 ± 0.57	2.99 ± 0.39



relative expression		2 h	24 h	48 h	72 h
GR1	5 µM Cd	1.12 ± 0.09	2.30 ± 0.01	1.61 ± 0.10	1.42 ± 0.09
	10 µM Cd	1.13 ± 0.07	2.86 ± 0.25	2.21 ± 0.22	2.32 ± 0.03
GSH1	5 µM Cd	0.85 ± 0.06	0.95 ± 0.04	1.09 ± 0.18	1.00 ± 0.04
	10 µM Cd	0.96 ± 0.05	1.46 ± 0.22	2.55 ± 0.43	1.88 ± 0.22
GSH2	5 µM Cd	0.72 ± 0.06	2.26 ± 0.25	1.35 ± 0.18	1.50 ± 0.27
	10 µM Cd	0.95 ± 0.23	4.29 ± 1.16	2.65 ± 0.20	1.88 ± 0.16

Fig. 3.5 *Concentrations of reduced (GSH) and oxidised (GSSG) forms of glutathione (nmol GSH equivalents g^{-1} FW) in roots (A) and leaves (B) of Arabidopsis thaliana exposed to 0 (□), 5 (■) or 10 (■) µM $CdSO_4$ for different time periods (0, 2, 24, 48, 72 h); n = 4 (* significantly different from the control condition, one-way ANOVA, P < 0.05). The table below displays expression levels of glutathione reductase 1 (GR1), glutamate cysteine ligase (GSH1) and glutathione synthetase (GSH2) relative to the corresponding control; n = 4 (significant up-regulation, ■; significant down-regulation, ■; one-way ANOVA, P < 0.05).*

Table 3.1 *Expression patterns of genes related to superoxide dismutase (SOD) regulation and catalase (CAT) relative to the control in roots and leaves of Arabidopsis thaliana exposed to 5 or 10 µM $CdSO_4$ for different durations (2, 24, 48, 72 h); n = 4 (significant up-regulation, ■; significant down-regulation, ■; one-way ANOVA, P < 0.05). Genes: microRNA398 (MIR398a and MIR398b/c), copper/zinc superoxide dismutase (CSD1 and CSD2), iron superoxide dismutase (FSD1), manganese superoxide dismutase (MSD1), copper chaperone for copper/zinc superoxide dismutase (CCS), copper chaperone (CCH), antioxidant protein 1 (ATX1) and catalase (CAT1, CAT2 and CAT3).*

Cd (µM)	ROOTS 2 h	ROOTS 24 h	ROOTS 48 h	ROOTS 72 h	genes	LEAVES 2 h	LEAVES 24 h	LEAVES 48 h	LEAVES 72 h	Cd (µM)
5	7.73 ± 1.13	21.24 ± 0.75	67.56 ± 2.04	40.40 ± 8.05	***MIR***	1.39 ± 0.23	5.58 ± 0.52	13.71 ± 4.38	8.48 ± 0.05	**5**
10	9.63 ± 1.14	29.19 ± 1.79	124.51 ± 22.88	78.54 ± 17.83	***398a***	0.83 ± 0.09	5.24 ± 1.27	17.27 ± 1.79	10.79 ± 0.02	**10**
5	1.23 ± 0.02	38.45 ± 0.33	59.00 ± 4.07	53.04 ± 2.72	***MIR***	1.32 ± 0.12	2.52 ± 0.46	8.25 ± 2.68	3.72 ± 0.89	**5**
10	1.18 ± 0.13	20.01 ± 0.32	33.97 ± 3.09	87.27 ± 41.23	***398b/c***	0.86 ± 0.03	1.81 ± 0.76	9.54 ± 1.88	9.55 ± 2.01	**10**
5	1.26 ± 0.17	0.56 ± 0.19	0.51 ± 0.05	0.47 ± 0.07	***CSD1***	1.01 ± 0.11	1.07 ± 0.20	0.56 ± 0.05	0.44 ± 0.06	**5**
10	0.99 ± 0.16	0.75 ± 0.11	1.35 ± 0.18	1.08 ± 0.22		1.25 ± 0.34	2.08 ± 0.66	0.46 ± 0.09	0.22 ± 0.06	**10**
5	1.09 ± 0.10	0.91 ± 0.23	0.63 ± 0.06	0.41 ± 0.03	***CSD2***	1.20 ± 0.16	0.40 ± 0.04	0.15 ± 0.02	0.19 ± 0.01	**5**
10	0.75 ± 0.14	0.85 ± 0.11	0.86 ± 0.11	0.55 ± 0.13		1.94 ± 0.47	0.44 ± 0.13	0.10 ± 0.01	0.04 ± 0.01	**10**
5	0.92 ± 0.12	14.99 ± 2.95	26.36 ± 5.86	23.18 ± 0.73	***FSD1***	1.60 ± 0.38	1.20 ± 0.09	6.19 ± 0.57	3.44 ± 0.09	**5**
10	0.72 ± 0.20	17.04 ± 2.01	16.98 ± 3.40	38.62 ± 9.33		1.15 ± 0.17	0.87 ± 0.17	3.06 ± 0.29	3.88 ± 0.33	**10**
5	1.05 ± 0.07	1.07 ± 0.09	0.98 ± 0.14	1.09 ± 0.14	***MSD1***	1.23 ± 0.06	0.89 ± 0.07	1.11 ± 0.02	1.15 ± 0.09	**5**
10	0.90 ± 0.05	1.36 ± 0.02	1.20 ± 0.04	1.16 ± 0.06		1.26 ± 0.15	1.28 ± 0.10	1.28 ± 0.08	1.22 ± 0.08	**10**
5	0.71 ± 0.15	0.51 ± 0.01	0.46 ± 0.15	0.31 ± 0.13	***CCS***	1.16 ± 0.23	0.32 ± 0.05	0.10 ± 0.00	0.22 ± 0.05	**5**
10	0.52 ± 0.24	0.40 ± 0.04	0.27 ± 0.04	0.17 ± 0.04		1.05 ± 0.03	0.27 ± 0.05	0.08 ± 0.01	0.07 ± 0.01	**10**
5	1.01 ± 0.14	3.76 ± 0.70	2.07 ± 0.09	2.50 ± 0.12	***CCH***	1.38 ± 0.13	0.92 ± 0.16	1.79 ± 0.13	1.51 ± 0.27	**5**
10	0.76 ± 0.11	3.28 ± 0.16	3.00 ± 0.52	5.27 ± 0.98		1.15 ± 0.13	0.81 ± 0.13	2.26 ± 0.03	2.78 ± 0.19	**10**
5	0.85 ± 0.10	1.66 ± 0.45	1.07 ± 0.17	0.89 ± 0.15	***ATX1***	1.23 ± 0.07	2.19 ± 0.30	2.29 ± 0.11	1.54 ± 0.18	**5**
10	0.99 ± 0.10	1.36 ± 0.24	1.32 ± 0.06	1.48 ± 0.19		1.36 ± 0.15	2.36 ± 0.33	2.34 ± 0.07	2.28 ± 0.12	**10**
5	0.81 ± 0.16	1.00 ± 0.06	0.70 ± 0.01	0.68 ± 0.18	***CAT1***	1.34 ± 0.26	1.57 ± 0.32	3.10 ± 0.12	1.83 ± 0.42	**5**
10	0.63 ± 0.22	4.75 ± 1.00	3.80 ± 0.38	2.22 ± 0.45		1.21 ± 0.08	2.95 ± 0.74	6.57 ± 1.55	5.95 ± 1.40	**10**
5	0.93 ± 0.10	0.92 ± 0.04	0.84 ± 0.09	0.51 ± 0.12	***CAT2***	1.43 ± 0.22	0.34 ± 0.04	0.51 ± 0.04	0.87 ± 0.05	**5**
10	0.73 ± 0.27	0.84 ± 0.05	0.47 ± 0.04	0.35 ± 0.01		1.44 ± 0.22	0.14 ± 0.05	0.29 ± 0.07	0.49 ± 0.11	**10**
5	0.86 ± 0.08	1.12 ± 0.04	1.34 ± 0.34	1.15 ± 0.04	***CAT3***	1.26 ± 0.10	1.03 ± 0.18	2.92 ± 0.20	3.16 ± 0.47	**5**
10	0.84 ± 0.30	1.82 ± 0.27	2.70 ± 0.23	2.74 ± 0.42		1.15 ± 0.12	1.09 ± 0.09	2.31 ± 0.22	4.75 ± 0.96	**10**

To investigate the correlation between SOD gene expression and activity, native PAGE and specific staining for SOD activity was performed (Fig. 3.6). The different bands were identified using isoform selective staining (data not shown). In roots, the band that represents FeSOD activity only appeared after 72 h of 10 µM Cd exposure, while an increase in FeSOD activity was observed in leaves. In contrast, CuZnSOD activity was reduced in a time- and dose-dependent manner in roots starting after 24 h, whereas in leaves CuZnSOD activity was decreased after 72 h. No visible changes in the activity of the manganese isoform of SOD (MnSOD) were detected.

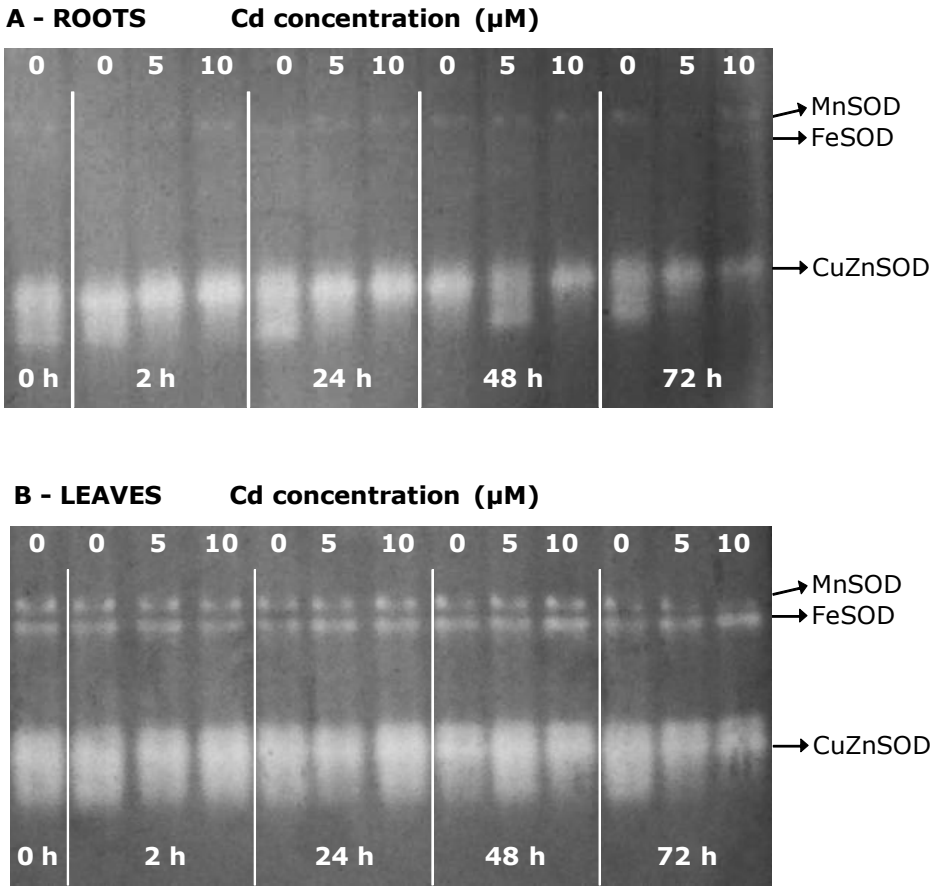
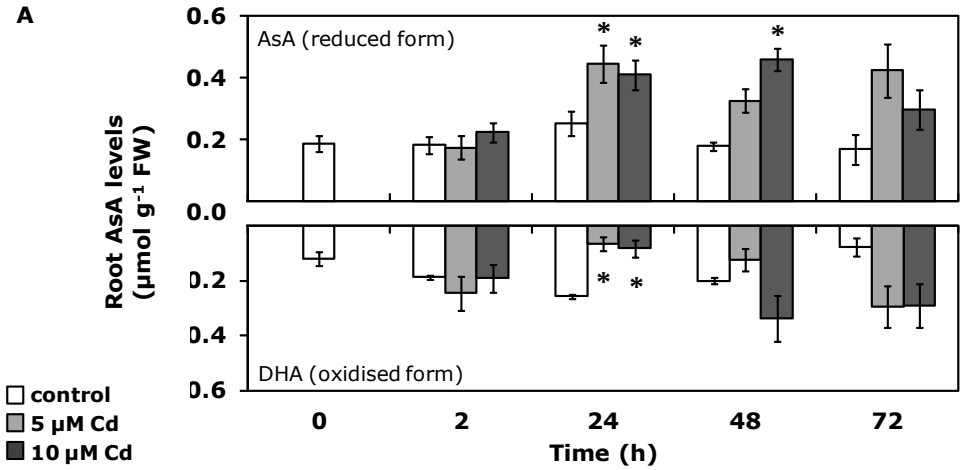


Fig. 3.6 *Superoxide dismutase (SOD) activity staining following non-denaturing PAGE in roots (A) and leaves (B) of Arabidopsis thaliana exposed to 0, 5 or 10 µM $CdSO_4$ for different time periods (0, 2, 24, 48, 72 h). Arrows indicate bands of the different SOD-isoforms: manganese SOD (MnSOD), iron SOD (FeSOD) and copper/zinc SOD (CuZnSOD).*

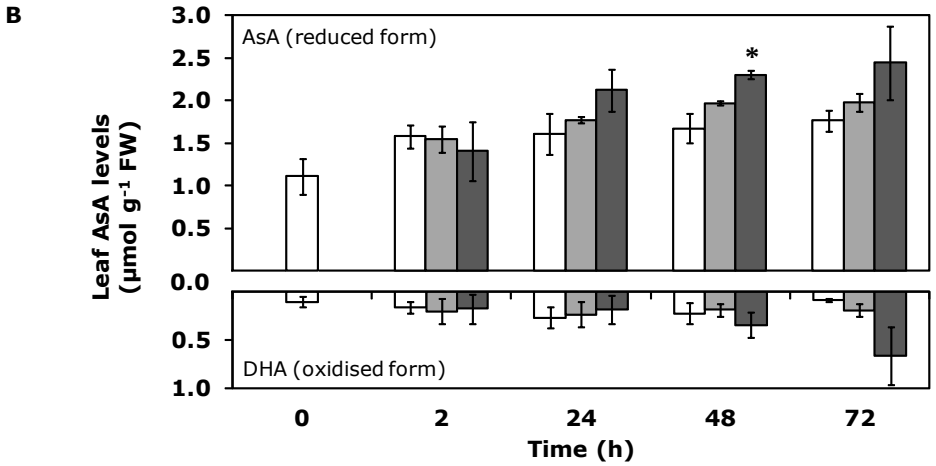
3.4.3 Activation of hydrogen peroxide-scavengers apart from glutathione

The products of $O_2^{\bullet-}$ dismutation by SOD are O_2 and H_2O_2. The latter can be detoxified by enzymes such as APx and CAT. Expression of cytosolic *APX* genes was determined in combination with the concentration and redox state of AsA (Fig. 3.7). Although *APX1* expression did not change during Cd stress, in both roots and leaves, transcript levels of *APX2* were significantly increased after 24 and 48 h of exposure to both Cd concentrations. The AsA levels did not show significant changes after 2 h. However, after 24 h the roots responded with a rise in AsA levels concomitant with a decrease in dehydroascorbate (DHA), the oxidised form of AsA, resulting in a more reduced state of the AsA pool ($P < 0.05$). The leaves were only responsive after 48 h of exposure to 10 µM Cd with increased AsA levels and after 72 h the AsA redox state was significantly shifted towards a more oxidised state ($P < 0.05$).

The three isoforms of CAT revealed a differential gene expression (Table 3.1). In roots, exposure to 10 µM Cd resulted in an elevation of gene transcripts of both *CAT1* and *CAT3* after 24 h, while a down-regulation of *CAT2* transcript levels was observed after 48 h. This differing regulation of CAT gene expression also appeared in leaves: *CAT1* and *CAT3* transcript levels were enhanced after 24 and 48 h exposure, respectively, and *CAT2* was down-regulated after 24 h.



relative expression		2 h	24 h	48 h	72 h
APX1	**5 µM Cd**	0.87 ± 0.089	0.65 ± 0.115	0.70 ± 0.068	0.74 ± 0.209
	10 µM Cd	0.83 ± 0.155	0.54 ± 0.064	0.86 ± 0.069	0.90 ± 0.20
APX2	**5 µM Cd**	0.74 ± 0.033	1.42 ± 0.43	0.56 ± 0.13	0.63 ± 0.136
	10 µM Cd	0.74 ± 0.045	4.12 ± 1.27	9.42 ± 1.59	7.06 ± 3.329



relative expression		2 h	24 h	48 h	72 h
APX1	**5 µM Cd**	0.82 ± 0.07	1.15 ± 0.305	1.15 ± 0.092	0.97 ± 0.056
	10 µM Cd	0.95 ± 0.162	1.49 ± 0.381	1.43 ± 0.08	1.09 ± 0.10
APX2	**5 µM Cd**	0.43 ± 0.00	4.01 ± 1.29	2.67 ± 0.40	1.07 ± 0.084
	10 µM Cd	0.79 ± 0.274	15.6 ± 4.74	4.37 ± 0.15	1.51 ± 0.188

Fig. 3.7 *Concentrations of reduced (AsA) and oxidised (DHA) forms of ascorbate (µmol g^{-1} FW) in roots (A) and leaves (B) of Arabidopsis thaliana exposed to 0 (□), 5 (■) or 10 (■) µM $CdSO_4$ for different time periods (0, 2, 24, 48, 72 h); n = 4 (* significantly different from the control condition, one-way ANOVA, P < 0.05). The table below displays expression levels of ascorbate peroxidase (APX1 and APX2) relative to the corresponding control; n = 4 (significant up-regulation, ■; significant down-regulation, ■; one-way ANOVA, P < 0.05).*

3.5 Discussion

The toxic effects of Cd on plant physiology and morphology are well-documented (Gallego *et al.* 1996; Kieffer *et al.* 2008; Keunen *et al.* 2011b; Cuypers *et al.* 2012a). Many sites of action have been identified but the spatiotemporal progression of the events taking place in Cd-affected tissues still remains unclear. Therefore, this study investigated the immediate and subsequent Cd-induced defence mechanisms in *A. thaliana* exposed to different environmental Cd concentrations. In accordance with previous work, exposure to Cd led to lower fresh weight (Fig. 3.2; Cuypers *et al.* 2009; Keunen *et al.* 2011b) and oxidative damage, measured by lipid peroxidation (Fig. 3.3; Cuypers *et al.* 2011). In order to cope with Cd, plants possess defence strategies in which GSH plays a central role as Cd chelator, antioxidant and signalling molecule (Jozefczak *et al.* 2012). Due to these multiple functions, this study focused on GSH and the sequence of its actions under Cd stress.

3.5.1 Cadmium-exposed Arabidopsis roots show a fast but glutathione-depriving phytochelatin response

In comparison to other non-essential metals, Cd has a high mobility and can quickly move across plasma membranes in plants (Schwartz *et al.* 2003). A well-known protection mechanism in plants is the capacity of roots to retain Cd ions and limit translocation to the shoots. Our data confirm a fast and strong accumulation of Cd in a dose- and time-dependent manner in roots (Fig. 3.1A). Once inside root cells, Cd forms complexes with ligands. Due to the high affinity of Cd for thiol groups, the cysteine containing non-protein thiols GSH and PC are major Cd chelators, crucial for Cd retention in roots (Rauser 2003). In order to provide sufficient thiols in roots, GSH was immediately allocated to PC production before *de novo* GSH biosynthesis was started. This is demonstrated by a constant total thiol pool consisting of newly formed PC2 (Fig. 3.4A) in combination with decreased GSH and GSSG levels (Fig. 3.5A) in roots after 2 h of Cd exposure. Although GSH biosynthesis was activated after 24 h, as indicated by increased total thiol levels and supported by the induction of biosynthesis genes, PC levels rose strongly, while GSH levels remained decreased. Roots exposed to 5 µM Cd were able to restore their GSH levels after 48 h. Exposure to the highest Cd concentration however, resulted in increased GSSG levels, shifting towards a GSSG:GSH ratio favouring the oxidised form,

while GSH content was still decreased. Increasing *GR1* transcripts, possibly to recycle the accumulating GSSG back to GSH, coincided with the restoration of GSH levels after 72 h and preventing further oxidation of the GSH pool.

This direct protection mechanism against Cd ions however, did not occur without consequences in the roots. Due to the initial GSH depletion triggered by the production of PCs, the direct and indirect ROS scavenging capacity of GSH is supposed to be reduced. The present data did not show an activation of alternative antioxidative defence mechanisms at the onset of GSH decrease after 2 h of Cd exposure. After 24 h however, the root antioxidant capacity was increased by multiple antioxidants. Firstly, the Cd-specific effects on SOD regulation at transcriptional (Table 3.1) and enzyme activity (Fig. 3.6A) levels are confirmed (Smeets *et al.* 2008a; Cohu *et al.* 2009; Cuypers *et al.* 2011): *FSD1* and *MIR398a-b/c* up-regulation and *CSD1/2* down-regulation, resulting in increased FeSOD activity and decreased CuZnSOD activities. The *CCS* transcripts, encoding the chaperones responsible for Cu delivery to CuZnSOD, are also targeted by *MIR*. As a result, Cu distribution to essential enzymes such as plastocyanin is stimulated via other chaperones (*i.e.* CCH or ATX1), while FeSOD is activated to compensate for CuZnSOD inactivation (Weigel *et al.* 2003; Sunkar & Zhu 2004; Semane *et al.* 2007; Huang *et al.* 2012). Secondly, in contrast to the constitutively expressed *APX1*, transcript levels of the stress-inducible *APX2* (Davletova *et al.* 2005) were increased after Cd exposure (Fig. 3.7A). This, in combination with a more reduced AsA pool due to both increased AsA and decreased DHA levels, indicates the stimulation of a functional ROS scavenging pathway using AsA as substrate. Finally, limited to roots exposed to the highest Cd concentration, CAT expression was enhanced in order to scavenge ROS in a substrate-independent manner (Table 3.1). To summarise, Cd-exposed roots showed a fast PC response at 2 h, followed by an efficient ROS neutralisation by pathways including GSH, FeSOD, AsA and CAT after 24 h.

3.5.2 Responses in leaves of cadmium-exposed Arabidopsis were delayed with respect to roots

Leaf Cd content increased within 24 h after Cd exposure and then stabilised, suggesting a state of equilibrium (Fig. 3.1B). Both Cd concentrations resulted in the same level of Cd accumulation in the leaves. Consequently, the Cd translocation factor was lower in plants exposed to the highest dose (Fig. 3.1C).

The latter confirms a recent study showing increasing Cd retention after exposure to increasing Cd concentrations (Nocito *et al.* 2011). As a consequence of limited Cd translocation, our data (summarised in Fig. 3.8) show delayed responses in leaves at different levels: (1) gene expression of the rate-limiting-enzyme GSH1 (Fig. 3.5B), together with SOD and SOD-related genes (Table 3.1); (2) biosynthesis of both metabolites PC (Fig. 3.4B) and AsA (Fig. 3.7B); and (3) the Cd-induced decrease in CuZnSOD activity (Fig. 3.6B) were all postponed in the leaves with respect to the roots.

This delay might protect the leaves from GSH depletion and oxidation as indicated by a simultaneous increase in GSH (Fig. 3.5B) and PC (Fig. 3.4B) levels after 24 h. At the same time, *GR1* expression was induced, which could lead to the observed decrease in GSSG levels. The sustained *GR1* up-regulation also contributed to the more reduced redox state of GSH in the leaves, in contrast to the roots. Additionally, the amount of non-protein thiols was higher in leaves than in roots since sulfur assimilation predominantly takes place in leaf chloroplasts, while roots largely depend on shoot-to-root transfer of GSH via the phloem (de Knecht *et al.* 1995; Davidian & Kopriva 2010). These high thiol levels in leaves reduce competition for GSH between Cd chelation and ROS detoxification. In addition to Cd retention in the roots and high reduced thiol levels in leaves, fast inter-organ redox signalling from the roots to the leaves have also been suggested as a protective mechanism during abiotic stress (Noctor *et al.* 2002; Foyer & Noctor 2005b). The transient decrease in root GSH content in Cd-exposed plants directly influenced the GSH:GSSG ratio, which is suggested to generate such a redox signal (Nocito *et al.* 2006). Moreover, previous studies show that Cd preferentially accumulates in leaf trichomes where large amounts of metals can be sequestered without damaging the cells (Isaure *et al.* 2006). In accordance with this statement, *MT2a* (Table A.3.3 in Appendices), which is known to be induced by metal and oxidative stress and primarily expressed in trichomes, was the most responsive gene of the protein thiols studied at transcript level (Garcia-Hernandez *et al.* 1998). Within the time frame of our study, all these features render the leaves very efficient in limiting excess ROS and free Cd levels in order to preserve plant survival. Plants exposed for 72 h to the highest dose however, showed decreasing PC levels (Fig. 3.4B). On the one hand, this might be explained by efficient Cd sequestration to the trichomes, which reduces the need for Cd chelation by PCs. Increasing GSH

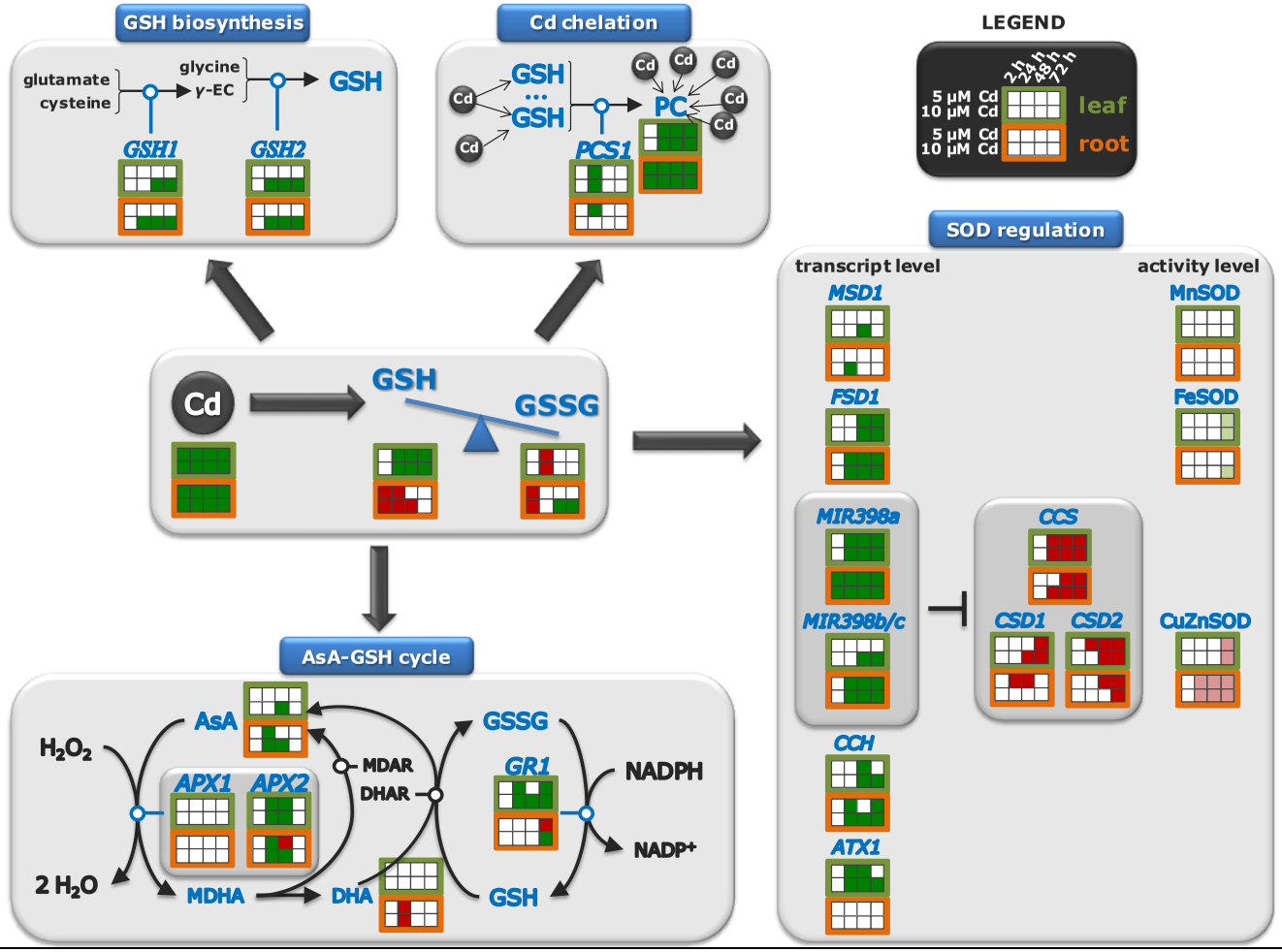
levels (Fig. 3.5B) are consistent with previous studies suggesting a role for GSH in trichomes as antioxidant and metal chelator (Gutierrez-Alcala *et al.* 2000). On the other hand, several toxic responses were observed in these plants: decreased or stunted fresh weight (Fig. 3.2), increased lipid peroxidation (Fig. 3.3) and oxidation of the root GSH redox state (Fig. 3.5A). Together with decreasing PC levels, these parameters might suggest system failure. In order to make more reliable conclusions about the latter, a long-term study is required.

3.5.3 Conclusion

The present study demonstrates a differential response of *Arabidopsis* leaves and roots to Cd. Additionally, GSH-related chelating and antioxidant capacity appear to be biphasic in roots. Previous findings concerning Cd retention and detoxification in roots by chelation with thiol compounds and subsequent sequestration were confirmed (Zhang *et al.* 2013). However, this protection mechanism leads to a dual time shift; sequential cellular responses were found within the roots and between organs. Firstly, although roots immediately used GSH for Cd chelation, resulting in a fast but GSH-depriving PC response, no alternative pathways were found to be activated at the same time. After 24 h however, multiple antioxidative pathways were activated in order to complement GSH's antioxidative functions during decreased GSH levels.

Secondly, a delayed response was observed in leaves with respect to roots due to limited Cd translocation. Together with high reduced leaf thiol contents and eventual signalling responses from the roots, the leaves were protected up to a certain level, allowing them time to activate their defence mechanisms. The rationale behind the observed biphasic response of the direct chelating and antioxidant capacity in roots is unknown. We hypothesise that Cd entering root cells is immediately scavenged by thiols, in particular PCs, at the expense of GSH. At the same time, a redox signal is suggested to be generated by a decreased GSH pool in combination with an altered GSH:GSSG ratio in order to increase the antioxidant capacity. Subsequently, the antioxidative defence system is activated and elevated ROS levels due to increasing Cd accumulation in roots can be efficiently neutralised. It remains challenging however, to further investigate and fully comprehend the complex network of interrelated processes (intracellular, intercellular, inter-organ) in order to keep the balance of GSHs functions as chelator, antioxidant and redox buffer.

Fig. 3.8 *Schematic overview of four studied processes, all activated under Cd stress: GSH biosynthesis, Cd chelation, SOD regulation and the AsA-GSH cycle. There are several indications that the GSH state, including both total GSH content and GSH:GSSG ratio, is involved in the stimulation of these pathways. Data of roots and leaves of Arabidopsis thaliana exposed to 5 or 10 µM $CdSO_4$ for different time periods (2, 24, 48, 72 h) are visualised (significant up-regulation, ■; significant down-regulation, ■; ANOVA). Data from in-gel activities are presented by ■ and ■ for increased and decreased SOD activity, respectively, according to visual quantification. Abbreviations: cadmium (Cd), glutathione (GSH), GSH disulfide (GSSG), γ-glutamylcysteine (γ-EC), γ-EC synthetase (GSH1), GSH synthetase (GSH2), phytochelatin (PC), PC synthase (PCS1), hydrogen peroxide (H_2O_2), ascorbate (AsA), AsA peroxidase (APX1, APX2), monodehydroascorbate (MDHA), monodehydroascorbate reductase (MDAR), dehydroascorbate (DHA), dehydroascorbate reductase (DHAR), GSH reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH, $NADP^+$), superoxide dismutase (SOD) isoforms: manganese (MSD1, MnSOD), iron (FSD1, FeSOD) and copper/zinc (CSD1, CSD2, CuZnSOD); microRNA398 (MIR398a, MIR398b/c), copper chaperones (CCH, ATX1, CCS). This pathway was also created in PathVisio to include annotations and bibliography of the genes, proteins and metabolites: http://www.wikipathways.org/index.php/Pathway:WP2579.*


GSH biosynthesis
glutamate
cysteine
glycine
γ-EC
GSH
GSH1
GSH2
Cd chelation
GSH
GSH
PC
PCS1
Cd
LEGEND
2 h
24 h
48 h
72 h
5 µM Cd
10 µM Cd
5 µM Cd
10 µM Cd
leaf
root
SOD regulation
transcript level
activity level
MSD1
MnSOD
FSD1
FeSOD
MIR398a
MIR398b/c
CCS
CSD1
CSD2
CuZnSOD
CCH
ATX1
Cd
GSH
GSSG
AsA-GSH cycle
AsA
H2O2
APX1
APX2
MDAR
DHAR
GSSG
GR1
NADPH
NADP+
2 H2O
MDHA
DHA
GSH

Appendices

Table A.3.1 *List of primers used in Reverse Transcription quantitative PCR (RTqPCR). Exon-Exon-junction (E-E-jn), untranslated region (UTR).*

Gene	TAIR: locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
ACTIN2	*AT3G18780*	Actin2	FW: CTTGCACCAAGCAGCATGAA REV: CCGATCCAGACACTGTACTTCCTT	Exon 2	68 bp	88.1%
APX1	*AT1G07890*	Ascorbate peroxidase1	FW: TGCCACAAGGATAGGTCTGG REV: CCTTCCTTCTCTCCGCTCAA	Exon 5	101 bp	96.3%
APX2	*AT3G09640*	Ascorbate peroxidase2	FW: TTGCTGTTGAGATCACTGGAGGA REV: TGAGGCAGACGACCTTCAGG	Exon 3	91 bp	90.9%
ATX1	*AT1G66240*	Homolog of antioxidant1	FW: TCACAGCTCCAACACATCCCT REV: CGATGGCGACTGCTCTCACT	Exon 2	91 bp	98.2%
CAT1	*AT1G20630*	Catalase1	FW: AAGTGCTTCATCGGGAAGGA REV: CTTCAACAAAACGCTTCACGA	E5-E6-jn	103 bp	97.6%
CAT2	*AT4G35090*	Catalase2	FW: AACTCCTCCATGACCGTTGGA REV: TCCGTTCCCTGTCGAAATTG	Exon 2	76 bp	98.3%
CAT3	*AT1G20620*	Catalase3	FW: TCTCCAACAACATCTCTTCCCTCA REV: GTGAAATTAGCAACCTTCTCGATCA	Exon 2	91 bp	95.6%
CCH	*AT3G56240*	Cu chaperone	FW: CTTTCACTGTCACCTTTTGCTCCT REV: TGCGTTGGTGCCGTCAATA	Exon 1	91 bp	91.8%
CCS	*AT1G12520*	Cu chaperone for CuZnSOD	FW: TTCACAGCATTAACACAACCCTCA REV: CAAGCCTTGTCGGTGGTTGA	Exon 4	91 bp	92.3%
CSD1	*AT1G08830*	CuZn superoxide dismutase1	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5	102 bp	93.8%
CSD2	*AT2G28190*	CuZn superoxide dismutase2	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6	101 bp	93.9%
FSD1	*AT4G25100*	Fe superoxide dismutase1	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4	101 bp	88.8%
GR1	*AT3G24170*	Glutathione reductase1	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15	101 bp	94.79%
GR2	*AT3G54660*	Glutathione reductase2	FW: GCCCAGATGGATGGAACAGAT REV: TAGGGTTGGAGAATGTTGGCG	Exon 5	91 bp	96.4%
GSH1	*AT4G23100*	γ-Glutamylcysteine synthetase	FW: CCCTGGTGAACTGCCTTCA REV: CATCAGCACCTCTCATCTCCA	Exon 5	101 bp	98.6%
GSH2	*AT5G27380*	Glutathione synthetase	FW: GGACTCGTCGTTGGTGACAA REV: TCTGGGAATGCAGTTGGTAGC	Exon 11	101 bp	92.6%

Table A.3.1 *Continued*

Gene	TAIR: locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
MIR172a[1]	*AT2G28056*	MicroRNA172a	Target: AGAAUCUUGAUGAUGCUGCAU	Lot Number: 0906007		
MIR398a[1]	*AT2G03445*	MicroRNA398a	Target: UGUGUUCUCAGGUCACCCCUU	Lot Number: 0905011		
MIR398b/c[1]	*AT5G14545*	MicroRNA398b/c	Target: UGUGUUCUCAGGUCACCCCUG	Lot Number: 0905005		
MSD1	*AT3G10920*	Mn superoxide dismutase1	FW: ATGTTTGGGAGCACGCCTAC REV: AACCTCGCTTGCATATTTCCA	Exon 5	101 bp	89.4%
MT1a (1)[2]	*AT1G07600*	Metallothionein1a	FW: AACTGTGGATGTGGCTCCTC REV: CAGTTACAGTTTGACCCACAGC	Exon 1	122 bp	90.4%
MT1a (2)[2]	*AT1G07590*	Tetratricopeptide repeat superfamily	FW: AGAGCTAGCGAGAACGTGGA REV: CCTACTCGAGCAAACGCTTC	Exon 4	93 bp	87.0%
MT1c	*AT1G07610*	Metallothionein1c	FW: GCATGGTCTCAAACCAAGGA REV: TACGCAACACAATGCCAAGT	Intron 2	96 bp	86.4%
MT2a	*AT3G09390*	Metallothionein2a	FW: ACCCTGACTTGGGATTCTCC REV: GCGTTGTTACTCTCCCCTGA	Exon 1	109 bp	90.2%
MT2b	*AT5G02380*	Metallothionein2b	FW: ACTCTTGTCCTCGGTGTTGC REV: TTGCACTTGCAGTCAGATCC	Exon 1	110 bp	81.2%
MT3	*AT3G15353*	Metallothionein3	FW: TCGACATCGTCGAGACTCAG REV: CACTTGCAATTTGCGTTGTT	Exon 1	85 bp	88.0%
PCS1	*AT5G44070*	Phytochelatin synthase1	FW: TGCCAAGGAGCTGAAATCTT REV: ACCGTGCCTTCAGAGTCATC	Exon 2	91 bp	95.1%
PCS2 [3]	*AT1G03980*	Phytochelatin synthase2	FW: CCATGGTTAGCCACCCGACT REV: TCTTCTTTATTCTCCTGGCATCGT	Exon 1	94 bp	109.8%
PPR	*AT5G55840*	Pentatricopeptide repeat superfamily	FW: AAGACAGTGAAGGTGCAACCTTACT REV: GTTTTTGAGTTGTATTTGTCAGAGAAAG	3'UTR	59 bp	81.5%
SAND	*AT2G28390*	SAND family	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13	61 bp	107.8%
Tip41-like	*AT4G34270*	Tip41-like	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn	61 bp	91.2%
UBC21	*AT5G25760*	Ubiquitin conjugating enzyme21	FW: CTGCGACTCAGGGAATCTTCTAA REV: TTGTGCCATTGAATTGAACCC	E3-E4-jn	61 bp	98.7%

[1] *MicroRNA primers were purchased from Applied Biosystems, their primer details were not revealed.*

[2] *Since AT1G07590 overlaps the MT1a gene completely, AT1G07590-specific primers were also developed to correct for its contribution to the MT1a gene expression.*

[3] *The PCS2 primer concentration was increased to 600 nM.*

Table A.3.2 *Reverse Transcription quantitative PCR (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines derived from Bustin et al. 2009.*

Sample/Template	
Source	*Arabidopsis thaliana* roots or shoots in a hydroponic culture
Method of preservation	harvest in liquid N_2 then stored at -80°C
Storage time	two weeks
Handling	frozen
Extraction method	columns: *mir*Vana miRNA Isolation Kit* (Ambion, Lennik, Belgium)
RNA: DNA-free	TURBO DNA-free Kit* (Ambion, Lennik, Belgium) intron-spanning primers and verification of single peak on melt curves
Concentration	NanoDrop®: ND-1000 Spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Microfluidics: Bioanalyzer* (Agilent Technologies, Waldbronn, Duitsland) for a representative subset of the samples
Assay optimisation/validation	
Accession number	see Table A.3.1
Amplicon details	exon location & amplicon size see Table A.3.1
Primer sequence	see Table A.3.1
In silico	Primer-BLAST
Empirical	primer concentration (300 nM unless stated otherwise) annealing temperature (60°C)
Priming conditions	random hexamer priming
PCR efficiency	dilution curves (slope, y-intercept & r^2, see Table A.3.1)
Linear dynamic range	samples are within the efficiency curve
RT/PCR	
Protocols	TURBO DNA-free Kit* (Ambion) Fast SYBR Green* (Applied Biosystems, Paisley, UK) see Materials and Methods
Reagents	see Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7900HT Sequence Detection Software, version 2.3 (Applied Biosystems, Foster city, California, USA, 2005)
Statistical justification	4 biological replicates linear mixed-effects one-way ANOVA and Tukey post-hoc adjustment for multiple comparison
Normalisation	3 reference genes selected using geNorm, version 3.5 (Centre for Medical Genetics, Ghent, Belgium, 2001-7)

*All procedures were performed according to the manufacturer's protocol.

Table A.3.3 *Expression patterns of metallothionein (MT) genes relative to the control in roots and leaves of three-week-old Arabidopsis exposed to 5 or 10 µM $CdSO_4$ during different time points (2, 24, 48, 72 h); n = 4 (significant up-regulation, ■; significant down-regulation, ■; one-way ANOVA, P < 0.05).*

Cd (µM)	ROOTS 2 h	ROOTS 24 h	ROOTS 48 h	ROOTS 72 h	genes	LEAVES 2 h	LEAVES 24 h	LEAVES 48 h	LEAVES 72 h	Cd (µM)
5	0.81 ± 0.16	1.00 ± 0.06	0.70 ± 0.01	0.68 ± 0.18	***CAT1***	1.34 ± 0.26	1.57 ± 0.32	3.10 ± 0.12	1.83 ± 0.42	**5**
10	0.63 ± 0.22	4.75 ± 1.00	3.80 ± 0.38	2.22 ± 0.45		1.21 ± 0.08	2.95 ± 0.74	6.57 ± 1.55	5.95 ± 1.40	**10**
5	0.93 ± 0.10	0.92 ± 0.04	0.84 ± 0.09	0.51 ± 0.12	***CAT2***	1.43 ± 0.22	0.34 ± 0.04	0.51 ± 0.04	0.87 ± 0.05	**5**
10	0.73 ± 0.27	0.84 ± 0.05	0.47 ± 0.04	0.35 ± 0.01		1.44 ± 0.22	0.14 ± 0.05	0.29 ± 0.07	0.49 ± 0.11	**10**
5	0.86 ± 0.08	1.12 ± 0.04	1.34 ± 0.34	1.15 ± 0.04	***CAT3***	1.26 ± 0.10	1.03 ± 0.18	2.92 ± 0.20	3.16 ± 0.47	**5**
10	0.84 ± 0.30	1.82 ± 0.27	2.70 ± 0.23	2.74 ± 0.42		1.15 ± 0.12	1.09 ± 0.09	2.31 ± 0.22	4.75 ± 0.96	**10**
5	0.46 ± 0.18	1.16 ± 0.37	0.33 ± 0.03	2.35 ± 1.39	***MT1a***	0.48 ± 0.15	1.38 ± 0.63	0.21 ± 0.03	1.12 ± 0.55	**5**
10	0.77 ± 0.33	2.25 ± 0.47	1.92 ± 0.61	1.46 ± 0.21		0.33 ± 0.14	1.70 ± 0.79	0.61 ± 0.08	1.98 ± 0.60	**10**
5	1.92 ± 0.27	0.73 ± 0.05	1.15 ± 0.38	1.02 ± 0.19	***MT1c***	1.22 ± 0.35	1.23 ± 0.49	1.53 ± 0.22	1.07 ± 0.10	**5**
10	1.60 ± 0.39	0.94 ± 0.06	0.88 ± 0.03	1.20 ± 0.18		2.66 ± 1.25	2.07 ± 0.80	5.42 ± 1.77	9.43 ± 4.42	**10**
5	0.76 ± 0.16	6.50 ± 1.96	2.82 ± 0.86	2.73 ± 0.66	***MT2a***	1.42 ± 0.05	3.12 ± 0.29	5.64 ± 0.49	2.65 ± 0.53	**5**
10	0.77 ± 0.20	9.97 ± 2.17	2.91 ± 0.62	4.96 ± 0.92		1.86 ± 0.35	2.91 ± 0.33	10.75 ± 0.16	10.02 ± 1.56	**10**
5	0.86 ± 0.09	1.14 ± 0.11	1.19 ± 0.16	0.73 ± 0.09	***MT2b***	1.05 ± 0.16	0.69 ± 0.08	0.51 ± 0.04	0.46 ± 0.05	**5**
10	0.71 ± 0.23	1.03 ± 0.03	0.93 ± 0.24	1.15 ± 0.23		1.27 ± 0.26	0.53 ± 0.09	0.45 ± 0.03	0.55 ± 0.07	**10**
5	2.46 ± 0.76	1.56 ± 0.52	1.76 ± 0.80	1.91 ± 0.60	***MT3***	1.95 ± 0.53	1.34 ± 0.58	0.55 ± 0.13	0.55 ± 0.09	**5**
10	1.71 ± 0.66	4.30 ± 2.32	4.74 ± 1.22	3.94 ± 0.31		3.21 ± 1.67	1.10 ± 0.40	0.72 ± 0.09	0.82 ± 0.30	**10**

Chapter 4

Deficiency in ascorbate is compensated by glutathione in cadmium-exposed *Arabidopsis* mutants but glutathione deficiency demands for multiple alternatives

Research article – submitted to New Phytologist

Deficiency in ascorbate is compensated by glutathione in cadmium-exposed *Arabidopsis* mutants but glutathione deficiency demands for multiple alternatives

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4.1 Abstract

Glutathione (GSH) and ascorbate (AsA) are abundantly occurring multifunctional antioxidants in plant cells. Both metabolites are kept in a generally reduced state to perform redox signalling and remove cadmium (Cd)-induced reactive oxygen species (ROS). In addition, GSH plays a major role in direct Cd detoxification by scavenging the free Cd ions. In this study, the importance of GSH and AsA after Cd exposure was investigated using mutant *Arabidopsis* plants containing only 30-45% of wild-type levels of GSH (*cad2-1*) or 40-50% of AsA (*vtc1-1*), together with the double mutant (*cad2-1 vtc1-1*). Both GSH deficient mutants exhibited an increased Cd sensitivity in comparison to the wild-type plants, as evidenced by the activation of alternative antioxidant pathways including AsA, superoxide dismutase (SOD) and catalase (CAT) in order to cope with Cd exposure. The *vtc1-1* plants however, demonstrated a decreased sensitivity to Cd, possibly due to an elevated antioxidant capacity (*i.e.* increased levels of GSH and transcripts of antioxidative enzymes) under control conditions. In conclusion, GSH appeared to be the key agent in fast root responses to toxic Cd exposure due to its dual function as antioxidant and chelator, while both GSH and AsA pathways were addressed in the leaves.

4.2 Introduction

Glutathione (GSH) and ascorbate (AsA) are widely distributed metabolites found at millimolar concentrations in plant cells. Both metabolites are involved in intertwined pathways related to growth, development, cellular defences and hence possess multiple essential functions, rendering them indispensible for normal metabolism as well as for cellular defences against stress (Smirnoff 2000; Noctor *et al.* 2012). Cadmium (Cd) is a non-essential element with a high toxicity towards human health and the environment (Nawrot *et al.* 2008). At cellular level, Cd indirectly causes oxidative stress, either via activation of pro-oxidants [e.g. NADPH oxidase, lipoxygenase (LOX)] or via inhibition of electron transport chains or antioxidative defence mechanisms (Heyno *et al.* 2008; Remans *et al.* 2010). Antioxidants are crucial to maintain the cellular redox balance and consist of both enzymes and metabolites. In the AsA-GSH cycle, ascorbate peroxidase (APx) neutralises hydrogen peroxide (H_2O_2) using the

successive oxidation and reduction of both metabolites (Mittler *et al.* 2004). Therefore, GSH and AsA are central metabolites in Cd-induced defence responses due to their capacity to scavenge reactive oxygen species (ROS) and hence their contribution to redox signalling. In addition, GSH is a major component in fast Cd chelation due to the high affinity of Cd for the thiol group of GSH, and as a precursor for phytochelatins (PCs) (Jozefczak *et al.* 2014).

Glutathione is synthesised in two ATP-dependent steps: peptide bond formation between *γ*-glutamate and cysteine by plastidial *γ*-glutamylcysteine synthetase (GSH1) and subsequent addition of glycine by glutathione synthetase (GSH2), which mainly occurs in the cytosol (Noctor *et al.* 2012). Cadmium toxicity has been shown to increase *GSH1* expression levels in order to meet the elevated GSH demand in plants for detoxification and survival (Xiang & Oliver 1998). The thiol group on cysteine is responsible for the direct chelation of Cd and allows GSH to participate in redox cycling (Jozefczak *et al.* 2012). The Cd-GSH complexes are sequestered in vacuoles (Cobbett & Goldsbrough 2002; Klein *et al.* 2006), away from sensitive sites in cells, protecting susceptible cysteine-rich proteins from binding with free Cd ions that could adversely affect their function (Noctor *et al.* 2012). Since GSH1 is the rate-limiting enzyme in GSH biosynthesis encoded by a single gene, mutational knockouts result in lethal phenotypes, whereas GSH deficient mutants due to decreased GSH1 activity are preferred to investigate the functions of GSH (Lim *et al.* 2011). The mutation in the GSH deficient *cad2-1 Arabidopsis* plant lies close to the active site in GSH1, causing reduced activity and hence reduced GSH levels (Hothorn *et al.* 2006). Although this mutant is more sensitive to Cd, it exhibits no phenotype under control conditions, suggesting that the residual GSH content (30-45% of wild-type levels) is sufficient for normal growth and metabolism (Cobbett *et al.* 1998).

The main biosynthetic pathway of AsA is the D-glucose/L-galactose (or Smirnoff-Wheeler) pathway consisting of nine cytosolic steps and a final step restricted to the mitochondria. Studies have indicated that guanosine diphosphate-D-mannose pyrophosphorylase (*GMP*) transcript levels alter upon Cd toxicity and it is the first enzyme in the AsA biosynthesis pathway. Since the *cyt1-2* mutation with a severe deficiency in GMP activity is lethal, the *vtc1-1* mutation with approximately 50% of wild-type GMP activity is favoured. However, the latter shows a slow-growth phenotype because AsA and its precursors are involved in

cell division, elongation and cell wall synthesis (Conklin *et al.* 1999; Veljovic-Jovanovic *et al.* 2001; Bielen *et al.* 2013).

Research has already indicated that antioxidants, both their concentrations and redox state, are crucial for plant defence against oxidative stress induced by metals like Cd (Noctor 2006; Cuypers *et al.* 2011). Given the above indications, we are interested in Cd-induced responses, specifically in plants with GSH and/or AsA deficiency. To investigate this, wild-type, *cad2-1*, *vtc1-1* and double mutant (*cad2-1 vtc1-1*) plants were exposed to environmentally realistic Cd concentrations.

4.3 Materials and methods

4.3.1 Plant material and growth conditions

Both wild-type (Lehle seeds, Round Rock TX, USA) and three mutant *Arabidopsis thaliana*, cv. Columbia seedlings were cultivated. The Cd-sensitive *cad2-1* mutant (Cobbett *et al.* 1998), donated by Dr. Christopher Cobbett (Melbourne University, Australia), has a mutation in *GSH1* (*AT4G23100*), which results in approximately 30% of wild-type levels of GSH. The ascorbate deficient *vtc1-1* mutant (Conklin *et al.* 1999), purchased from Nottingham *Arabidopsis* Stock Centre (NASC ID: N8326, UK), has a mutation in *GMP* (*AT2G39770*), which results in approximately 40-50% of wild-type levels of AsA. The double mutant *cad2-1 vtc1-1* (Clerkx *et al.* 2004) was kindly provided by Dr. Mark Aarts (Wageningen University, the Netherlands). All genotypes were surface sterilised and grown under the following conditions: 12 h/22°C day, 12 h/18°C night regime, 65% relative humidity and 170 µmol m^{-2} s^{-1} light at the rosette level delivered by cool white fluorescent lamps (L 140W/20SA, Osram, Augsburg).

Plants were cultivated in hydroponics for three weeks before exposure to 5 µM $CdSO_4$ (Chapter 3). After 24 h, root samples harvested for Cd determination were washed with ice-cold 10 mM $Pb(NO_3)_2$ (15 min, 4°C) and rinsed in distilled water, while leaves were washed in distilled water. Cadmium concentrations were measured after weighing and digestion of dried material (60°C, two weeks) in 70-71% HNO_3 using a heat block via inductively coupled plasma-optical emission spectrometry (ICP-OES 710, Agilent Technologies, Australia). For the

other experiments, samples were collected, snap frozen in liquid nitrogen and stored at -80°C for further analysis.

To analyse leaf growth, plants were cultivated on round Petri dishes (90 mm). The medium consisted of a 50-fold dilution of the Gamborg B5 medium [macronutrients: 0.5 mM KNO_3, 0.02 mM $MgSO_4$, 0.02 mM $CaCl_2$, 0.022 mM $NaH_2PO_4.2H_2O$, 0.02 mM $(NH_4)_2SO_4$; and micronutrients: 1.183 µM $MnSO_4.H_2O$, 0.090 µM KI, 0.97 µM H_3BO_3, 0.14 µM $ZnSO_4.7H_2O$, 0.1 µM $CuSO_4.5H_2O$, 0.021 µM $Na_2MoO_4.2H_2O$, 0.002 µM $CoCl_2.2H_2O$, 2 µM $FeNO_3.9H_2O$] supplemented with 0.05% (w/v) MES, 0.5% (w/v) sucrose, 0.7% (w/w) agar and 5 µM $CdSO_4$. After two weeks of germination, leaf surfaces were analysed using Optimas (Optimas Corporation, version 6.1, UK). Root growth analysis was performed on square Petri dishes (120 mm x 120 mm) containing the same medium, except 1% (w/w) agar was used (Remans *et al.* 2012b). After one week, plants were transferred to medium without sucrose, containing the following range of Cd concentrations: 0, 0.5, 1.5 and 5 µM. Root growth was analysed after one week of exposure in scanned images using the Optimas image analysis software (MediaCybernetics).

4.3.2 Analysis of gene expression

Samples were disrupted under frozen conditions using two stainless steel beads and the Retch Mixer Mill MM 2000 (Retsch, Germany). RNA was extracted using the *mir*Vana miRNA Isolation Kit (Ambion, Belgium) and briefly incubated in gDNA wipeout buffer (42°C, 2 min) to effectively remove contaminating gDNA before reverse transcription was carried out using the Quantitect Reverse Transcription Kit (Qiagen, the Netherlands) with an equal amount of RNA input (1 µg). After a tenfold dilution in 1/10 diluted TE-buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), the cDNA was stored at -20°C. Quantitative PCR (qPCR) was performed with an ABI PRISM 7500 Fast Real-Time PCR System using Fast SYBR Green chemistry according to the manufacturer's instructions (Applied Biosystems, Belgium). Relative gene expression was calculated as $2^{-\Delta Cq}$ and was normalised to the expression of three reference genes: a SAND family protein (SAND, *AT2G28390*), yellow-leaf-specific gene8 (YLS8, *AT5G08290*) and an F-box protein (F-box, *AT5G15710*) (Remans *et al.* 2008). Gene-specific primers (300 nM, Table A.4.1 in Appendices) were designed via de Primer Express v2.0 software (Applied Biosystems). Table A.4.2 in Appendices shows the Reverse

Transcription quantitative PCR (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines (Bustin *et al.* 2009).

To analyse the mature microRNAs, a final purification of total RNA, enriched for small RNAs, was performed (*mir*Vana miRNA Isolation Kit, Ambion). Twenty ng microRNA was primed with three specific primers (*MIR172a*, *MIR398a* and *MIR398b/c*) according to the manufacturer's instructions (Multiplex RT for TaqMan MicroRNA Assay, Ambion). After a tenfold dilution in RNase-free water, the cDNA was stored at -20°C. Gene expression was based on TaqMan Universal PCR (Ambion) in an ABI PRISM 7500 Fast Real-Time PCR System using 9600 Emulation Mode according to the manufacturer's instructions and normalised against *MIR172a*. All primers for microRNAs (62.5 nM, Table A.4.1 in Appendices) were purchased from Applied Biosystems in Belgium.

4.3.3 Hierarchical clustering of gene expression data

To recognise potential sample-related patterns during Cd exposure in four *Arabidopsis thaliana* genotypes (wild-type, *cad2-1*, *vtc1-1* and *cad2-1 vtc1-1*), hierarchical clustering analysis was performed using GenEx software (v6, MultiD Analyses AB, Sweden). This analysis was based on raw gene expression values and the "Average linkage" algorithm, defining the distance between conditions as the average of distances between all pairs of individuals in all groups. Distances between the measures were calculated via the Euclidian Distance Measure. Heat maps were constructed to compare expression levels between different genes and samples.

4.3.4 Metabolite measurements

Total GSH and AsA determinations were performed on reverse-phase HPLC. In brief, frozen plant tissue (100-150 mg) was ground in liquid nitrogen and homogenised in 0.7 ml (roots) or 1 ml (leaves) 6% (w/v) meta-phosphoric acid. After centrifugation (10 min, 16000 g, 4°C), antioxidants were separated at 30-40°C on a reversed phase type Polaris 3 C_{18} column (3 µm particle size, 100 mm x 4.6 mm, Varian, USA) with an isocratic flow of 1 ml min^{-1} of the elution buffer (25 mM K/PO_4, pH 3). Components were quantified using a homemade electrochemical detector (glassy carbon working electrode and Schott pt 62 reference electrode). A diode array detector (SPD-M10AVP, Shimadzu, the

Netherlands) was used to confirm the purity and identity of the peaks. In order to measure total metabolite content, samples were reduced by incubation in 400 mM Tris and 200 mM DTT (15 min in dark) and adjusted pH between 6 and 7. Prior to HPLC analysis, the pH was lowered by a four-fold dilution in elution buffer.

Phytochelatins were also analysed by reverse-phase HPLC but first, thiols were derivatised with monobromobimane (mBrB) (Huguet *et al.* 2012). Briefly, 25 mg aliquots of material ground in 0.1% trifluoroacetic acid, 6.3 mM diethylene triamine pentaacetic acid (DTPA) (pH ≤ 1) and 10 µM N-acetyl-l-cysteine (NAC, internal standard) were filtered and incubated at 45°C with 280 µM mBrB, 125 mM HEPPS (pH 8.2) and 4 mM DTPA (30 min in dark). Thiols were separated at 37°C on a Nova-Pak C_{18} column (Waters, USA) and eluted with a slightly concave gradient of water and methanol. Fluorescent compounds were identified with Ellman's reagent using a Waters 474 fluorescence detector. Quantification was based on NAC and GSH standards and corrected for derivatisation efficiency.

4.3.5 Enzyme measurements

Frozen tissues (150 mg roots and 200 mg leaves) were ground in liquid nitrogen and agitated (30 min, 4°C) in 2 ml ice-cold 0.1M Tris-HCl buffer (pH 7.8) containing 5 mM EDTA, 5 mM dithioerythritol, 1% polyvinylpyrrolidone and 1% Nonidet P40. After centrifugation (48 384 *g*, 30 min, 4°C), the proteins in the supernatant were first precipitated in 40% and subsequently in 80% ammonium sulphate. The resulting pellet was resuspended in 1.75 ml 25 mM Tris-HCl (pH 7.8) and purified in PD-10 desalting columns (GE Healtcare, UK) by centrifugation (1 578 *g*, 2 min, 4°C). The protein extracts were stored at -80°C for further analysis. Due to APx instability, another extraction method was used for APx activity measurements: frozen tissues (100 mg roots and 200 mg leaves) were homogenised with 5 mg insoluble polyvinylpyrrolidone in 2 ml ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM DDT, 1 mM EDTA and 10 mM Na-AsA and centrifuged (10 min, 16 100 *g*, 4°C).

The enzyme activities were measured spectrophotometrically in the supernatant at 25°C. Determination of superoxide dismutase (SOD, E.C. 1.15.1.1) activity was based on the inhibition of cytochrome *c* at 550 nm (McCord & Fridovich 1969). Analysis of glutathione reductase (GR, E.C. 1.6.4.2) capacity was based

on the reduction of GSSG in the presence of NADPH at 340 nm and catalase (CAT, E.C. 1.11.1.6) activity was measured at 240 nm (Bergmeyer *et al.* 1974). Peroxidase (E.C. 1.11.1.9) activity was determined using guaiacol or syringaldazine as a substrate. Guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD) capacity were measured at 436 and 530 nm, respectively (Bergmeyer *et al.* 1974; Imberty *et al.* 1984). Glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49) activity was measured at 340 nm (Bergmeyer *et al.* 1974). Ascorbate peroxidase (E.C. 1.11.1.11) capacity measurement was based on AsA oxidation, which was measured at 298 nm (Gerbling *et al.* 1984).

4.3.6 Statistical analysis

Apart from the root growth experiment, all data were analysed with general linear ANOVA models (Verbeke & Molenberghs 2000). Normal distribution was tested using the following tests: Shapiro-Wilk, Kolmogorov-Smirnov, Craner-von Mises and Anderson-Darling. Transformations were applied when necessary to approximate normality and all gene expression data were log-transformed. Tukey post-hoc adjustment was used to correct for multiple comparison. All statistical analyses were performed using SAS 9.2 (SAS Institute Inc.). Since normality could not be reached for data in the root growth experiment, a non-parametric Kruskal-Wallis test was used in Excel (Microsoft Office 2007). Data are mean values ± standard error (SE) and significance was set at 5% ($P < 0.05$).

4.4 Results

4.4.1 Phenotypic changes in Arabidopsis thaliana exposed to cadmium

No significant differences in fresh weight (Table 4.1) or rosette phenotype (Fig. 4.1) were noticed between wild-type and *cad2-1* plants under control conditions. Both AsA deficient mutants (*vtc1-1* and *cad2-1 vtc1-1*) however, had a significantly lower fresh weight and their cotyledons showed slight leaf chlorosis. After 24 h of Cd exposure, only root weight was significantly reduced in wild-type plants, whereas *cad2-1* mutants showed decreased weight of both, roots and leaves. Although no Cd-induced effects were detected on fresh weight in the AsA deficient mutants, their cotyledons were chlorotic in comparison with the other genotypes.

Fresh weight (mg plant^{-1})	0 µM Cd	5 µM Cd
	LEAVES	
wild-type	77.25 ± 3.64^{AB}	72.95 ± 3.41^{A}
cad2-1	91.25 ± 3.61^{B}	69.70 ± 3.68^{AC}
vtc1-1	54.79 ± 3.06^{D}	65.87 ± 2.75^{AD}
cad2-1 vtc1-1	53.79 ± 2.81^{D}	57.39 ± 2.65^{CD}
	ROOTS	
wild-type	35.97 ± 2.61^{a}	26.38 ± 2.75^{bc}
cad2-1	32.21 ± 2.09^{ab}	19.10 ± 1.29^{c}
vtc1-1	17.39 ± 1.46^{c}	22.16 ± 1.44^{c}
cad2-1 vtc1-1	10.26 ± 0.87^{d}	11.40 ± 0.69^{d}

Table 4.1 *Fresh weight (mg FW plant^{-1}) of roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 h in hydroponics. Statistical significance is expressed using lower (roots) and upper (leaves) case letters; n = 25 (two-way ANOVA, P < 0.05).*

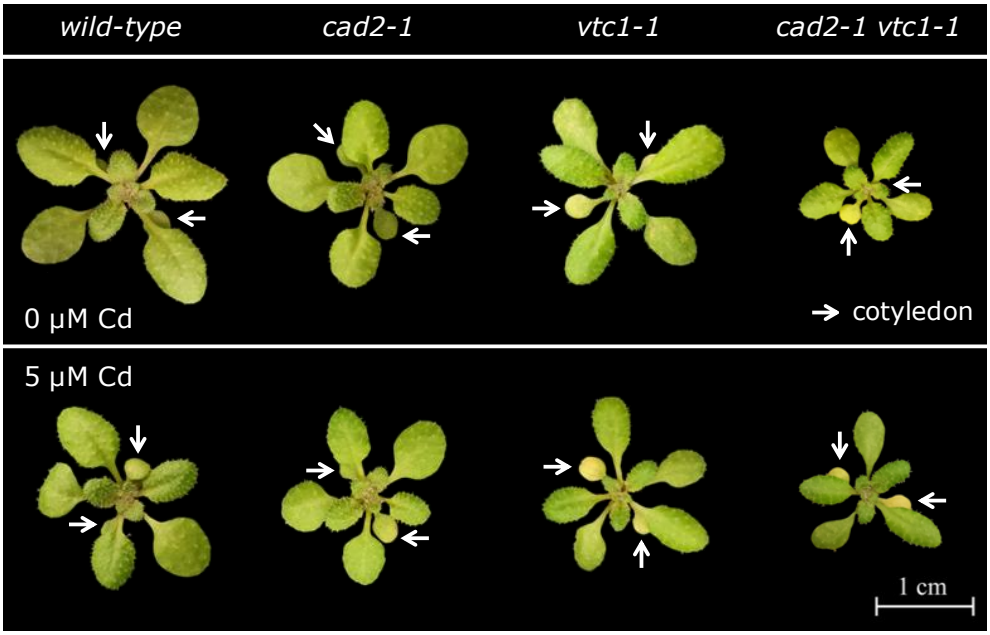


Fig. 4.1 *Representative pictures of the rosette appearance of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 h in hydroponics; n = 28. Cotyledons are indicated by an arrow.*

Glutathione and AsA contents were verified in the mutants: *cad2-1* and *cad2-1 vtc1-1* mutants displayed decreased GSH levels (25-40% of wild-type levels), while *vtc1-1* and *cad2-1 vtc1-1* mutants showed decreased AsA levels (30-55%) (Fig. 4.2). After exposure to Cd, the double mutant contained a higher Cd concentration in its roots than the other genotypes (Fig. 4.3A). In the leaves however, all mutants showed lower Cd concentrations than the wild-type plants. Therefore, the ratio of Cd concentration in the leaves to the roots (*i.e.* the translocation factor) was significantly lower in all mutants compared to the wild-type plants with the lowest translocation factor in the GSH deficient mutants (*cad2-1* and *cad2-1 vtc1-1*) (Fig. 4.3B).

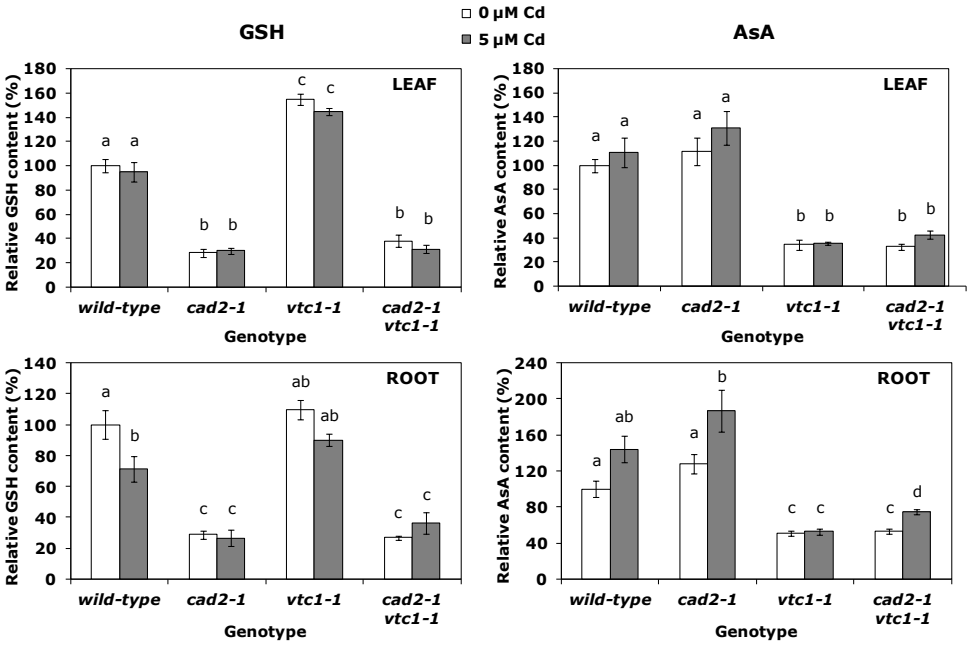


Fig. 4.2 *Concentrations of total glutathione (GSH) and total ascorbate (AsA) relative to the wild-type control (100%) in leaves and roots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 (□) or 5 (■) µM $CdSO_4$ for 24 h in hydroponics. Statistical significance is expressed using lower case letters; n = 4 (two-way ANOVA, P < 0.05).*

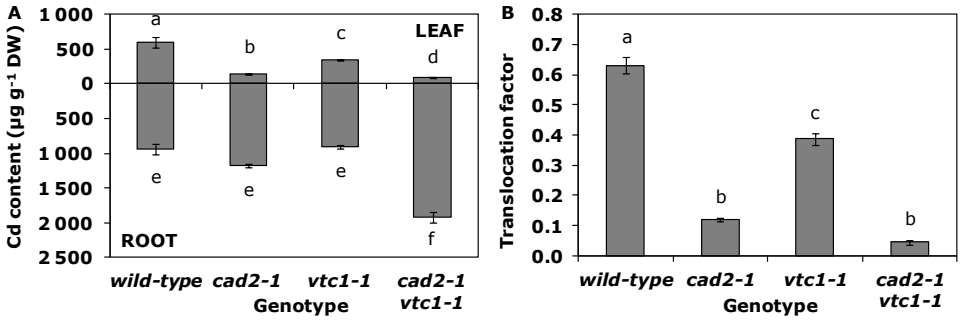


Fig. 4.3 *(A) Root and leaf Cd contents (µg g^{-1} DW) and (B) the corresponding translocation factors from roots to shoots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 5 µM $CdSO_4$ for 24 h in hydroponics. Statistical significance is expressed using lower case letters; n = 5 (A, two-way ANOVA; B, one-way ANOVA; P < 0.05).*

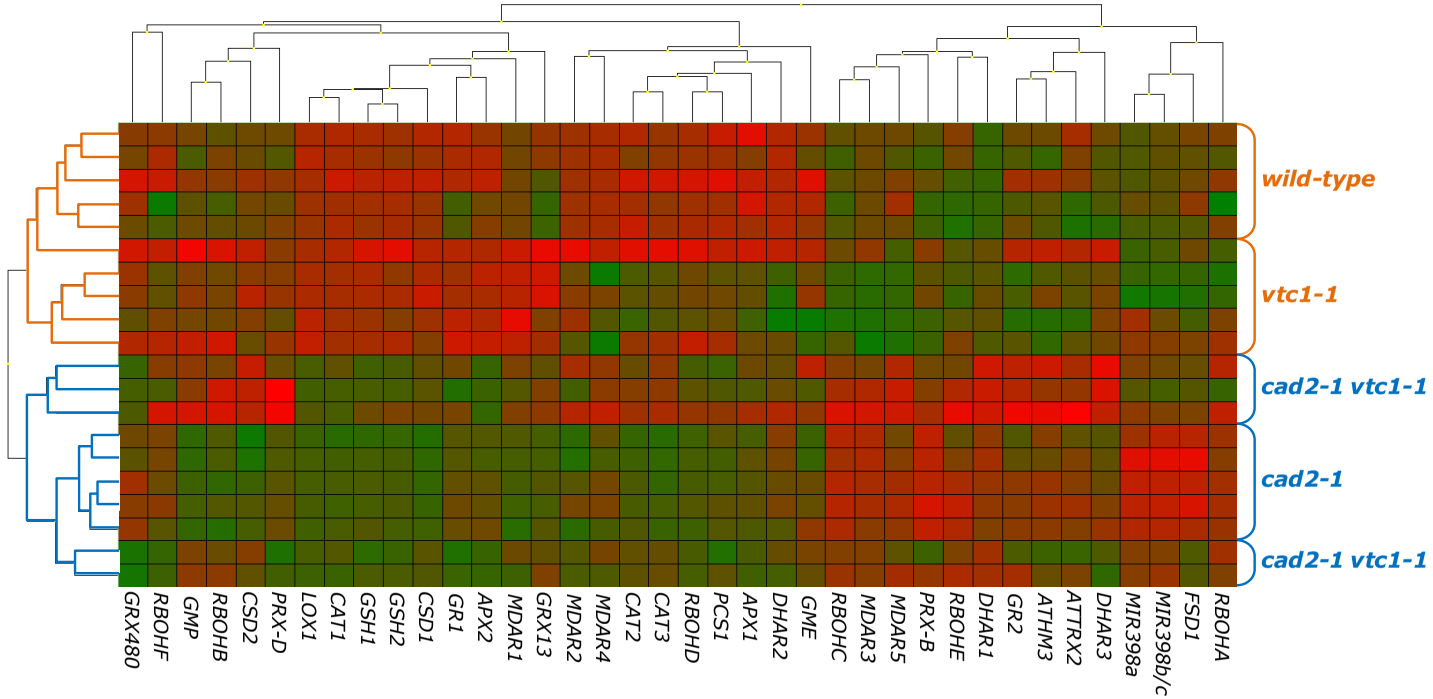
4.4.2 Glutathione and ascorbate deficiency induce a distinct gene expression profile

Four major groups of genes were investigated at transcriptional level (Table 4.2). The genes of GSH and AsA biosynthetic pathways listed in the first group represent key genes in the production of both metabolites together with the polymerisation of GSH to phytochelatin (PC) by phytochelatin synthase (*PCS*). The second group consists of antioxidative genes involved in superoxide ($O_2^{\bullet-}$) scavenging and the third group contains antioxidative genes that neutralise H_2O_2, *i.e. CAT* and genes related to the AsA-GSH cycle. The latter includes *APX*, monodehydroascorbate reductases (*MDAR*), dehydroascorbate reductases (*DHAR*) and *GR*. Also members of the redoxin family are able to reduce H_2O_2: glutaredoxins (*GRX*), peroxiredoxins (*PRX*) and thioredoxins (*TRX*) and are included in this group. The last group represents pro-oxidative genes including NADPH oxidase (*RBOH*) and *LOX* isoforms. Overall, no significant genotype effects were observed in the mutants under control conditions, unless stated otherwise. In roots, a general induction of practically all investigated genes was apparent in both GSH deficient mutants after Cd exposure, whereas only some antioxidative related genes (*FSD1*, *GRX13* and/or *ATTRX2*) and most pro-oxidative genes were induced in roots of wild-type and *vtc1-1* plants. Interestingly, the well-known Cd-induced SOD signature, *i.e.* increased *MIR398a-b/c* transcripts targeting *CSD1/2* transcripts and hence cause *CSD* down-regulation (Cuypers *et al.* 2011), was only apparent in wild-type and *vtc1-1* plants, while both GSH deficient mutants showed increased *CSD1/2* transcripts despite the up-regulation of *MIR398a-b/c*. Heat maps, generated from all gene expression data, visualise hierarchical agglomerate clustering combining both genes and genotypes. In Cd-exposed roots, two clusters were created: (1) wild-type and *vtc1-1* plants and (2) *cad2-1* and *cad2-1 vtc1-1* plants (Fig. 4.4).

Table 4.2 *Gene expression patterns relative to the control condition of the corresponding genotype (1.00) in roots and leaves of four Arabidopsis thaliana genotypes: wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1. Plant roots were exposed to 5 µM $CdSO_4$ for 24 h in hydroponics; n = 5 (significant up-regulation, ■; significant down-regulation, ■; one-way ANOVA, P < 0.05). Genes: γ-glutamylcysteine synthetase (GSH1), glutathione synthetase (GSH2), phytochelatin synthase (PCS1), GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose 3',5'-epimerase (GME), iron superoxide dismutase (FSD1), copper/zinc superoxide dismutase (CSD1/2), microRNA398 (MIR398a-b/c), catalase (CAT1-3), ascorbate peroxidase (APX1/2), monodehydroascorbate reductase (MDAR1-5), dehydroascorbate reductase (DHAR1-3), glutathione reductase (GR1/2), glutaredoxin (GRX13/480), peroxiredoxin (PRX-B/D), thioredoxin (ATTRX2 and ATHM3), NADPH oxidase (RBOHA-F), lipoxygenase (LOX1/2). Due to low abundance in the respective organ (*), these genes could not be analysed.*

ROOTS				GENES	LEAVES			
wild-type	***cad2-1***	***vtc1-1***	***cad2-1 vtc1-1***		***wild-type***	***cad2-1***	***vtc1-1***	***cad2-1 vtc1-1***
GSH/AsA biosynthesis								
0.92 ± 0.02	3.32 ± 0.12	0.90 ± 0.04	4.79 ± 0.16	***GSH1***	1.31 ± 0.12	2.64 ± 0.19	0.86 ± 0.03	0.87 ± 0.15
1.04 ± 0.06	3.29 ± 0.08	1.04 ± 0.09	5.52 ± 0.28	***GSH2***	1.50 ± 0.10	4.34 ± 0.67	0.98 ± 0.04	1.26 ± 0.33
1.17 ± 0.07	3.50 ± 0.18	1.52 ± 0.15	4.24 ± 0.30	***PCS1***	4.50 ± 0.75	1.49 ± 0.15	2.21 ± 0.44	1.14 ± 0.26
1.15 ± 0.10	2.08 ± 0.10	1.17 ± 0.18	3.12 ± 0.23	***GMP***	1.71 ± 0.03	1.04 ± 0.10	1.20 ± 0.09	0.85 ± 0.03
0.94 ± 0.04	1.86 ± 0.08	1.03 ± 0.06	1.96 ± 0.18	***GME***	0.95 ± 0.10	1.01 ± 0.05	1.28 ± 0.31	0.86 ± 0.16
$O_2^{\bullet-}$ scavenging								
7.26 ± 0.59	2.43 ± 0.24	7.39 ± 1.10	9.56 ± 0.50	***FSD1***	1.70 ± 0.36	0.87 ± 0.14	1.22 ± 0.20	0.51 ± 0.13
0.51 ± 0.02	2.50 ± 0.08	0.50 ± 0.05	3.08 ± 0.16	***CSD1***	1.45 ± 0.18	2.63 ± 0.20	0.74 ± 0.17	0.40 ± 0.05
0.76 ± 0.03	1.75 ± 0.10	0.63 ± 0.04	1.73 ± 0.14	***CSD2***	0.48 ± 0.14	0.35 ± 0.06	0.55 ± 0.15	0.22 ± 0.03
4.72 ± 0.08	3.32 ± 0.43	5.03 ± 0.78	3.35 ± 0.26	***MIR398a***	1.95 ± 0.37	1.62 ± 0.11	1.43 ± 0.31	1.76 ± 0.50
10.6 ± 0.84	2.53 ± 0.25	6.15 ± 0.84	3.36 ± 0.11	***MIR398b/c***	1.40 ± 0.26	1.24 ± 0.12	1.81 ± 0.25	1.87 ± 0.32
H_2O_2 scavenging/AsA-GSH cycle								
0.87 ± 0.05	6.29 ± 0.35	0.93 ± 0.02	9.36 ± 1.11	***CAT1***	1.54 ± 0.27	2.17 ± 0.16	0.91 ± 0.08	0.62 ± 0.08
1.18 ± 0.15	2.26 ± 0.09	0.96 ± 0.19	2.15 ± 0.12	***CAT2***	1.17 ± 0.14	1.07 ± 0.14	1.16 ± 0.21	0.49 ± 0.11
0.97 ± 0.11	2.02 ± 0.10	1.51 ± 0.35	2.43 ± 0.23	***CAT3***	1.10 ± 0.19	1.37 ± 0.25	1.31 ± 0.16	0.77 ± 0.14
0.84 ± 0.05	1.61 ± 0.03	0.94 ± 0.06	2.61 ± 0.15	***APX1***	2.03 ± 0.28	2.56 ± 0.25	1.06 ± 0.09	0.63 ± 0.14
2.28 ± 0.93	14.7 ± 2.62	0.59 ± 0.08	49.1 ± 5.26	***APX2***	2.60 ± 0.65	44.8 ± 10.8	1.93 ± 0.38	4.29 ± 2.59
1.02 ± 0.05	3.61 ± 0.21	0.91 ± 0.06	2.65 ± 0.24	***MDAR1***	1.41 ± 0.09	1.88 ± 0.10	1.11 ± 0.05	1.19 ± 0.13
1.20 ± 0.02	2.13 ± 0.16	0.94 ± 0.08	2.15 ± 0.20	***MDAR2***	7.33 ± 0.87	10.4 ± 1.32	2.18 ± 0.39	2.18 ± 0.50
0.97 ± 0.11	0.51 ± 0.05	0.97 ± 0.18	0.52 ± 0.07	***MDAR3***	210 ± 31.9	41.6 ± 8.09	9.82 ± 2.19	1.13 ± 0.26
1.34 ± 0.14	1.60 ± 0.12	1.14 ± 0.18	1.95 ± 0.13	***MDAR4***	1.30 ± 0.03	1.10 ± 0.07	1.16 ± 0.06	0.67 ± 0.11
0.70 ± 0.06	1.05 ± 0.08	0.82 ± 0.03	1.27 ± 0.07	***MDAR5***	0.65 ± 0.04	0.95 ± 0.05	0.81 ± 0.03	0.58 ± 0.12
1.06 ± 0.02	1.17 ± 0.05	0.92 ± 0.04	0.96 ± 0.05	***DHAR1***	0.65 ± 0.08	0.52 ± 0.03	0.85 ± 0.03	0.70 ± 0.13
1.24 ± 0.11	1.63 ± 0.13	0.98 ± 0.18	1.87 ± 0.17	***DHAR2***	2.31 ± 0.12	4.13 ± 0.64	1.64 ± 0.16	1.14 ± 0.18
0.71 ± 0.02	1.13 ± 0.05	0.80 ± 0.03	1.89 ± 0.23	***DHAR3***	3.61 ± 1.02	1.64 ± 0.26	1.18 ± 0.14	1.01 ± 0.25
1.42 ± 0.10	3.64 ± 0.11	1.04 ± 0.04	5.07 ± 0.36	***GR1***	3.38 ± 0.22	3.78 ± 0.13	1.43 ± 0.23	0.75 ± 0.12
0.96 ± 0.05	1.37 ± 0.07	0.91 ± 0.06	1.53 ± 0.07	***GR2***	0.84 ± 0.09	0.69 ± 0.02	0.93 ± 0.08	0.79 ± 0.04
3.75 ± 0.87	4.13 ± 0.24	0.41 ± 0.11	5.24 ± 0.44	***GRX13***	6.32 ± 1.26	10.5 ± 2.39	2.65 ± 0.61	0.61 ± 0.16
0.22 ± 0.03	8.54 ± 1.30	1.46 ± 0.26	4.76 ± 0.31	***GRX480***	224 ± 38.0	46.7 ± 8.48	8.88 ± 3.60	1.94 ± 0.85
1.00 ± 0.03	0.66 ± 0.03	1.04 ± 0.08	2.09 ± 0.13	***PRX-B***	0.61 ± 0.10	0.37 ± 0.02	0.78 ± 0.03	0.52 ± 0.11
0.76 ± 0.05	1.43 ± 0.05	0.67 ± 0.04	4.70 ± 0.77	***PRX-D***	1.61 ± 0.11	1.86 ± 0.05	1.20 ± 0.13	0.80 ± 0.10
1.16 ± 0.03	1.74 ± 0.04	1.08 ± 0.02	2.39 ± 0.07	***ATTRX2***	2.59 ± 0.23	2.90 ± 0.16	1.39 ± 0.17	0.96 ± 0.09
0.93 ± 0.02	1.30 ± 0.05	0.99 ± 0.03	1.86 ± 0.07	***ATHM3***	1.97 ± 0.12	2.16 ± 0.21	1.36 ± 0.13	1.07 ± 0.08
ROS production								
1.52 ± 0.12	2.56 ± 0.10	1.57 ± 0.13	3.79 ± 0.44	***RBOHA****				
2.30 ± 0.23	2.83 ± 0.23	1.43 ± 0.22	3.71 ± 0.43	***RBOHB****				
1.38 ± 0.07	0.83 ± 0.05	1.38 ± 0.13	1.67 ± 0.13	***RBOHC***	61.5 ± 9.15	95.4 ± 34.4	8.03 ± 4.39	15.1 ± 9.14
1.30 ± 0.14	6.82 ± 0.33	2.17 ± 0.55	7.10 ± 0.86	***RBOHD***	4.20 ± 0.25	5.73 ± 0.77	2.39 ± 0.43	1.56 ± 0.27
2.11 ± 0.16	2.21 ± 0.18	1.82 ± 0.12	3.10 ± 0.20	***RBOHE***	6.18 ± 1.00	9.44 ± 0.61	2.57 ± 0.45	2.02 ± 0.45
1.61 ± 0.14	2.61 ± 0.10	1.43 ± 0.06	3.39 ± 0.19	***RBOHF***	4.15 ± 0.12	2.14 ± 0.18	1.67 ± 0.36	0.90 ± 0.14
2.64 ± 0.29	49.1 ± 2.62	1.57 ± 0.25	70.6 ± 6.22	***LOX1***	2.15 ± 0.58	9.14 ± 1.32	0.99 ± 0.09	0.70 ± 0.09
				LOX2*	10.8 ± 2.08	3.14 ± 0.47	1.81 ± 0.14	1.42 ± 0.43

Fig. 4.4 *Heat map representations of the gene expression data collected from the roots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 5 µM $CdSO_4$ for 24 h in hydroponics (n = 5). Hierarchical clustering of genes is shown on the top and bottom, genotype clustering is visualised on the left and right. Green shaded boxes indicate high and red shaded boxes low gene expression. Genes: γ-glutamylcysteine synthetase (GSH1), glutathione synthetase (GSH2), phytochelatin synthase (PCS1), GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose 3',5'-epimerase (GME), iron superoxide dismutase (FSD1), copper/zinc superoxide dismutase (CSD1/2), microRNA398 (MIR398a-b/c), catalase (CAT1-3), ascorbate peroxidase (APX1/2), monodehydroascorbate reductase (MDAR1-5), dehydroascorbate reductase (DHAR1-3), glutathione reductase (GR1/2), glutaredoxin (GRX13/480), peroxiredoxin (PRX-B/D), thioredoxin (ATTRX2 and ATHM3), NADPH oxidase (RBOHA-F), lipoxygenase (LOX1).*



Although the leaves showed no clear gene clusters, some significant genotype effects were observed on leaf transcript levels in both AsA deficient mutants under control conditions: *MDAR3* and *GRX480* were significantly up-regulated (Fig. 4.5). After Cd exposure, leaves of wild-type and *cad2-1* plants activated nearly all investigated pro-oxidative genes. Also GSH biosynthesis genes were induced in both genotypes and only one AsA biosynthesis gene in wild-type plants. Apart from *CSD1*, *CAT1* and *APX2*, which were only induced in the leaves of *cad2-1* plants, the other GSH- and AsA-related genes were induced by both genotypes. The leaves of *vtc1-1* plants however, showed less transcriptional activation and the double mutant only showed down-regulation of some antioxidative genes.

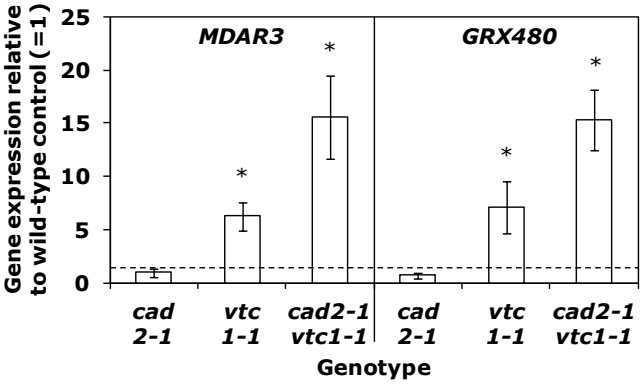


Fig. 4.5 *Expression of monodehydroascorbate reductase3 (MDAR3) and glutaredoxin480 (GRX480) in leaves of three Arabidopsis thaliana mutants (cad2-1, vtc1-1 and cad2-1 vtc1-1) under control conditions. Gene expression is relatively expressed to wild-type control (1: dashed line, n = 5, one-way ANOVA, significance level: * P < 0.05).*

4.4.3 Metabolite contents in Arabidopsis thaliana genotypes

After Cd exposure, roots of wild-type plants showed a significant decrease in GSH levels, while in leaves the metabolite concentrations remained constant (Fig. 4.2). Although no effect of Cd was detected in *vtc1-1* plants, leaf GSH levels were significantly elevated under control conditions. Concerning the AsA levels, only GSH deficient mutants showed changes in their root AsA content, which was significantly increased after exposure to Cd.

Since the heat maps clearly indicated a distinct behaviour for GSH deficiency after Cd exposure (Fig. 4.4), a more elaborate study was performed on wild-type and *cad2-1* plants. First, both genotypes were grown on agar plates to compare their sensitivity towards Cd. Exposure to the lowest Cd concentration

(0.5 µM) resulted in an immediate decrease in *cad2-1* root growth, while root growth of wild-type plants was only significantly decreased at the highest Cd concentration, (Fig. 4.6A). In the leaves, a significant growth reduction was observed in *cad2-1* plants after exposure to 5 µM Cd, while wild-type leaf growth only showed a decreasing trend (Fig. 4.6B).

Phytochelatin production was determined in roots and leaves of both genotypes (Fig. 4.7). Under control conditions, no PCs were detected except in roots of wild-type plants, which contained low levels of PC2 and PC3. After exposure to Cd, wild-type plants produced PCs up to PC5 and *cad2-1* mutants up to PC4 in both plant organs. However, total PC levels of *cad2-1* only reached about 20 and 15% of wild-type PC levels in roots and leaves, respectively.

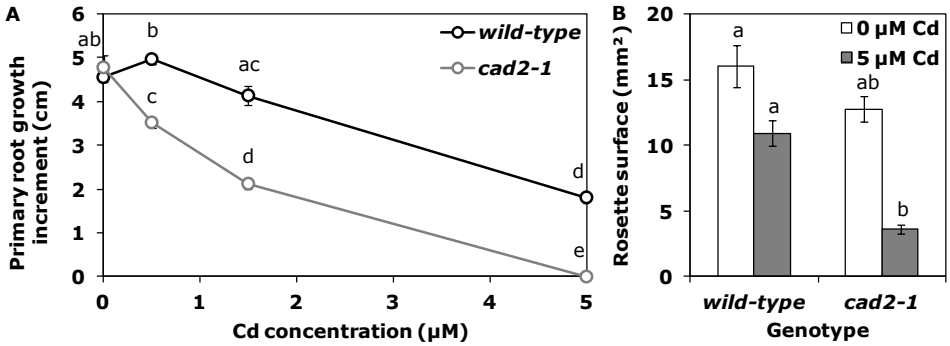


Fig. 4.6 *(A) The increment of primary root growth (cm) during one week of two Arabidopsis thaliana genotypes [wild-type (○) and cad2-1 (○)] after transfer to agar plates containing 0, 0.5, 1.5 and 5 µM $CdSO_4$; n = 20. (B) The rosette surface (mm²) of two week old Arabidopsis thaliana plants (wild-type and cad2-1) grown on agar plates supplemented with 0 (□) or 5 (■) µM $CdSO_4$; n = 3. Statistical significance is expressed using lower case letters (two-way ANOVA, P < 0.05).*

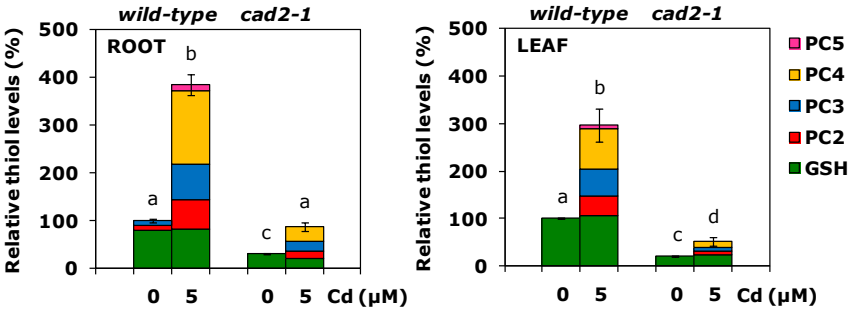


Fig. 4.7 *Profiling the total non-protein thiol content including glutathione [GSH, (■)] and phytochelatins [PC2 (■), PC3 (■), PC4 (■) and PC5 (■)] in two Arabidopsis thaliana genotypes (wild-type and cad2-1) in roots and leaves exposed to 0 or 5 µM $CdSO_4$ for 24 h in hydroponics. Thiol content is expressed relative to the wild-type control (100%). Statistical significance in total thiol levels is expressed using lower case letters; n = 4 (two-way ANOVA, P < 0.05).*

4.4.4 Genotype-specific changes in enzyme activity profiles

Activities of two groups of enzymes were analysed in wild-type and *cad2-1* genotypes; enzymes involved in (1) the antioxidative defence (SOD, CAT, GR, APx and G6PDH) and (2) cell wall lignification (GPOD and SPOD) (Cuypers *et al.* 2002). Although no genotype-effects were detected in enzyme activities under control conditions, exposure to Cd resulted in genotype-specific changes in activity (Fig. 4.8). Concerning enzymes involved in lignification, a general increase or increasing trend was observed in both organs of wild-type and *cad2-1* genotypes. All antioxidative enzyme activities, except APx, however, showed opposite trends in roots of wild-type *versus cad2-1* plants. Activities tended to decrease or remain stable in roots of wild-type plants, whereas root activities in *cad2-1* mutants tended to increase. This was most pronounced in SOD and CAT activities since SOD activity was significantly increased in roots of *cad2-1* plants and CAT activity was significantly decreased in roots of Cd-exposed wild-type plants. In leaves, these opposite trends were only present for SOD activity and to a lower extent than in roots.

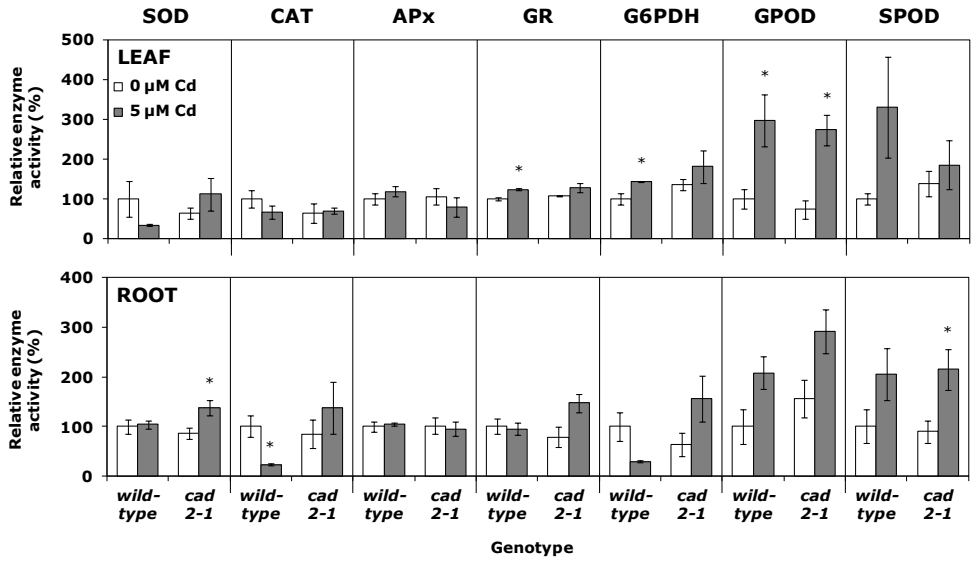


Fig. 4.8 *Enzyme activities relative to the wild-type control (100%) in leaves and roots of two Arabidopsis thaliana genotypes (wild-type and cad2-1) exposed to 0 (□) or 5 (■) µM $CdSO_4$ for 24 h in hydroponics; n = 4 (***** significantly different from the control condition of the corresponding genotype, one-way ANOVA, P < 0.05). Enzymes: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD).*

4.5 Discussion

Metals like Cd are well known for their disturbance of plant morphology, physiology and the cellular redox homeostasis. In order to reach a new cellular equilibrium upon metal exposure, plants increase their defence mechanisms (Kieffer *et al.* 2008; Cuypers *et al.* 2011; Keunen *et al.* 2011b). This study investigates the importance of two abundantly occurring multifunctional metabolites, GSH and AsA, in Cd toxicity by exposing three-week-old *Arabidopsis thaliana* mutants deficient in one or both metabolites to an environmentally realistic Cd concentration of 5 µM during 24 h (Krznaric *et al.* 2009). Glutathione on the one hand, is essential for its Cd chelating as well as antioxidant properties via the thiol group on cysteine (Jozefczak *et al.* 2012; Noctor *et al.* 2012). Ascorbate on the other hand, is the most abundant reducing substrate for H_2O_2 detoxification either directly or via the AsA-GSH cycle. Both metabolites interact with multiple enzymes involved in the antioxidative defence (Foyer & Noctor 2011; Bielen *et al.* 2013), of which the majority is included in the transcriptional profile of this study.

4.5.1 Changes in ascorbate content alter plant growth and metabolism under control conditions

Different phenotypic characteristics were apparent between unexposed wild-type and AsA deficient plants. Both mutants, containing about 40% of wild-type AsA levels (Fig. 4.2), exhibited slow-growth phenotypes (Table 4.1), which is consistent with the function of AsA and its precursors in cell wall synthesis, cell division and elongation (Veljovic-Jovanovic *et al.* 2001; Pastori *et al.* 2003; Pavet *et al.* 2005). Additionally, AsA deficient mutants have been associated with elevated levels of abscisic acid (ABA), a cell survival hormone, which induces metabolic arrest in order to sustain stress resistance (Pastori *et al.* 2003; Barth *et al.* 2006). Although still poorly understood, the inverse relationship between growth and defence is crucial to ensure plant reproduction under stress conditions. A second phenotypic symptom of AsA deficiency that we observed were signs of chlorosis (Fig. 4.1), which has been attributed to ABA-induced early senescence in *vtc1-1* mutants (Conklin & Barth 2004; Barth *et al.* 2006). These phenotypic observations support the fact that AsA deficiency challenges the fitness of plants, even in the absence of additional stress.

We hypothesise that *vtc1-1* mutants are continuously primed and hence prepared to cope with additional stresses like Cd toxicity. Previous studies suggest that AsA deficient plants are more resistant to pathogen infections due to a decreased threshold of ROS sensitivity causing premature activation of plant innate defence responses (Barth *et al.* 2004; Conklin & Barth 2004; Pavet *et al.* 2005; Mukherjee *et al.* 2010). Our data present evidence for an increased defence capacity in the leaves of *vtc1-1* mutants under control conditions, which might render them less sensitive to Cd-induced oxidative stress. First, AsA deficient leaves showed increased expression of the AsA-reducing *MDAR3* (Fig. 4.5), indicating that the mutants invested more in a highly reduced AsA pool. Since AsA must be fully reduced for ROS scavenging activity, not only the rate of AsA synthesis but also recycling via DHAR and MDAR is critical (Conklin & Barth 2004). Our finding of elevated *MDAR3* transcripts supports the absence of increased oxidative stress in *vtc1-1* plants despite their low AsA levels (Veljovic-Jovanovic *et al.* 2001; Pastori *et al.* 2003; Pavet *et al.* 2005). The second gene that was up-regulated, *GRX480* (Fig. 4.5), belongs to a family of GSH-dependent redox enzymes that protect thiol-containing proteins from irreversible oxidation and regulates the protein thiol/disulfide status, a primary mechanism in redox signalling (Meyer *et al.* 2008). Together with AsA, both genes have also been associated with ABA signalling, which is involved in numerous physiological processes including growth and defence systems (Pastori *et al.* 2003; Conklin & Barth 2004; Barth *et al.* 2006; Mukherjee *et al.* 2010). More specifically, ABA-mediated plant responses have been associated with WRKY33 signalling, a cascade involving several redox-related genes including *MDAR3* and *GRX480*. This complex transcriptional network is intertwined with other phytohormones and signalling cascades, which is still poorly understood. However, our data indicate that low AsA conditions lead to extensive cross-talk between these different signalling cascades, which possibly contributes to the increasing *MDAR3* and *GRX480* expression (Jiang & Deyholos 2009; Birkenbihl *et al.* 2012; Zander *et al.* 2012). Finally, at the level of metabolites, leaves of *vtc1-1* plants possessed elevated GSH contents in comparison to wild-type plants under control conditions (Fig. 4.2). Glutathione, as key compound in redox signalling and antioxidative responses has been demonstrated before to enhance the basal resistance level of *vtc1-1* mutants (Veljovic-Jovanovic *et al.* 2001; Pavet *et al.*

2005). Additionally, GSH plays an important role in Cd chelation via PC production (Jozefczak *et al.* 2012; Jozefczak *et al.* 2014). Increased basal GSH levels might indicate a higher potential to produce PCs, which in turn contributes to elevated Cd sequestration and hence less Cd-induced ROS production and damage (de Knecht *et al.* 1995; Zhu *et al.* 1999b; Li *et al.* 2006). Another mechanism in plants to chelate Cd is via enhanced cell wall lignification (Schreiber *et al.* 1999; Cuypers *et al.* 2002). Previous studies have associated elevated peroxidase activity in *vtc1-1* plants with increased pathogen resistance due to extensive cell wall crosslinking (Veljovic-Jovanovic *et al.* 2001; Colville & Smirnoff 2008), which might also contribute to an apoplastic transport barrier against Cd. Consistent with the elevated leaf antioxidant, signalling and chelating capacity in *vtc1-1* mutants under control conditions, their fresh weight was unaltered upon exposure to Cd, in contrast to the Cd-induced decrease or decreasing trend in roots and leaves of wild-type plants, respectively (Table 4.1). Equal Cd concentrations in roots (Fig. 4.3) resulted in a similar transcript profile of wild-type and *vtc1-1* plants (Table 4.2, Fig. 4.4), suggesting that AsA deficiency in roots had no significant effect on Cd-induced plant responses. In leaves however, *vtc1-1* plants were generally less responsive to Cd exposure than wild-type plants at the transcript level. Limited Cd translocation to the leaves in *vtc1-1* mutants, in combination with an elevated basal antioxidant and chelating capacity, partially explains their priming for additional stress and hence a minor response to Cd exposure with respect to wild-type plants.

4.5.2 Changes in glutathione content alter plant growth and responses under cadmium exposure

In line with previous studies, our data demonstrate that 70% reduction in GSH levels had no influence on the normal fitness of *Arabidopsis* plants (Table 4.1, Fig. 4.1, 4.6; Howden *et al.* 1995a; Cobbett *et al.* 1998; Parisy *et al.* 2007). It has been shown that GSH deficient mutants maintain high mitochondrial GSH levels, which are suggested to be important for plant development and growth, thus allowing a phenotype similar to wild-type plants (Zechmann *et al.* 2008). Additional studies suggest that there is a threshold GSH level, below which developmental effects are observed, but this is lower than the GSH levels in *cad2-1* plants (Wójcik & Tukiendorf *et al.* 2011).

In agreement with our previous findings, roots of wild-type plants responded to Cd exposure with a fast but GSH-depriving PC production (Fig. 4.2, 4.7) and increased lignification (Fig. 4.8), both contributing to Cd chelation and/or reduction of free cellular Cd levels. Additionally, early SOD responses complemented these chelation processes before H_2O_2 scavenging was activated (Table 4.2, Fig. 4.8, Jozefczak *et al.* 2014). Opposite regulations of the SOD-related genes resulted in a net zero response at SOD activity level in wild-type plants. This, in combination with unaltered expression and activity of the other antioxidants studied, supported that wild-type plants were able to cope with the Cd levels present at this time. Transcriptional activation of two major thiol-redox enzymes (*GRX13* and *ATTRX2*) that complement the GSH system in redox signalling (Meyer *et al.* 2008), suggests that efficient acclimation mechanisms focused primarily on GSH and its functions as chelator and signalling molecule. Cadmium additionally induced GSH- and AsA-related genes in the leaves of wild-type plants, as previously reported (Semane *et al.* 2007; Smeets *et al.* 2008a; Sobrino-Plata *et al.* 2014). This suggests that both metabolites are required to support Cd chelation on the one hand (GSH) as well as antioxidant and signalling capacities (AsA and GSH) on the other hand in order to cope with the present Cd levels.

In contrast to *vtc1-1* mutants, *cad2-1* plants are not equipped with elevated basal defence capacities and even decreased pathogen resistance has been attributed to these mutants (Parisy *et al.* 2007). Recent findings showed that in contrast to AsA deficient mutants, GSH deficient mutants were not able to compensate low metabolite levels and hence developed Cd-induced symptoms (Howden *et al.* 1995a; Koffler *et al.* 2014), which is also demonstrated in our study via the reduction of fresh weight (Table 4.1), rosette surface (Fig. 4.1, 4.6B) and root growth (Fig. 4.6A). In addition, a severe lack of Cd chelation via PCs (Fig. 4.7) and of ROS neutralisation via GSH (directly or via the AsA-GSH cycle, Table 4.2, Fig. 4.2), resulted in early activations of alternative antioxidant pathways in GSH deficient roots exposed to Cd in comparison to wild-type plants (Jozefczak *et al.* 2014). On the one hand, the SOD pathway is strongly stimulated, which is observed in the overruling of the negative *MIR398* regulation on *CSD* expression, leading to increased total SOD activity (Fig. 4.8). On the other hand, the two major H_2O_2 scavenging pathways were activated: up-regulation of *CAT* expression, preventing a drop in CAT activity, and *APX* expression, accompanied by elevated AsA levels and expression of its reducing

genes. This, in combination with more severe Cd-induced morphological effects in roots of *cad2-1* mutants, definitely supports that GSH is the primary factor in fast root responses to Cd toxicity (Jozefczak *et al.* 2014). The leaves of *cad2-1* mutants also responded to Cd exposure with activation of alternative antioxidant pathways, but to a minor extent than in roots.

4.5.3 Both metabolites differentially influence the outcome of cadmium-induced oxidative challenges

Cadmium toxicity is characterised by a cellular redox imbalance, whether this oxidative challenge leads to acclimation or damage, depends on plants' redox signalling and subsequent detoxification capacity (Seth *et al.* 2012). The redox couples, GSH, AsA and NADPH have been suggested to determine the cellular redox state and regulate downstream signalling cascades (Noctor 2006). Two major Cd detoxification strategies were investigated: chelation and the antioxidative defence (for a schematic overview, see Fig. 4.9).

The gene expression profile of roots corresponds to a Cd-induced oxidative burst consisting of up-regulated pro-oxidative genes, *i.e. RBOH* and *LOX* (Table 4.2) (Horemans *et al.* 2007; Remans *et al.* 2010; Keunen *et al.* 2013b). This burst is accompanied by GSH redox signalling and immediate activation of PCS by Cd-GSH complexes (Fig. 4.7). A minor role for AsA in fast root responses upon Cd exposure is supported by the fact that AsA deficient mutants show an equal transcriptional response as the roots of wild-type plants. Furthermore, the high Cd sensitivity in the GSH deficient mutant was similar to the one observed in roots of mutants deficient in both GSH and AsA. Leaves also showed up-regulation of pro-oxidative genes, demonstrating an oxidative challenge induced by Cd. In contrast to the roots however, they activated GSH-, AsA- and NADPH-related pathways (Table 4.2, Fig. 4.8), indicating that the three major redox couples come into play. On the one hand, leaves increased their chelating capacity via PC production, both at transcriptional (*i.e. PCS1* induction) and posttranslational level (*i.e.* increased GSH levels). On the other hand, leaf antioxidant capacity was stimulated via both GSH- and AsA-related genes. In combination with an elevated Cd sensitivity in GSH deficient leaves and severe morphological disturbances in leaves deficient in GSH and AsA, these data suggest that the leaves require both metabolites in order to acclimate to Cd exposure.

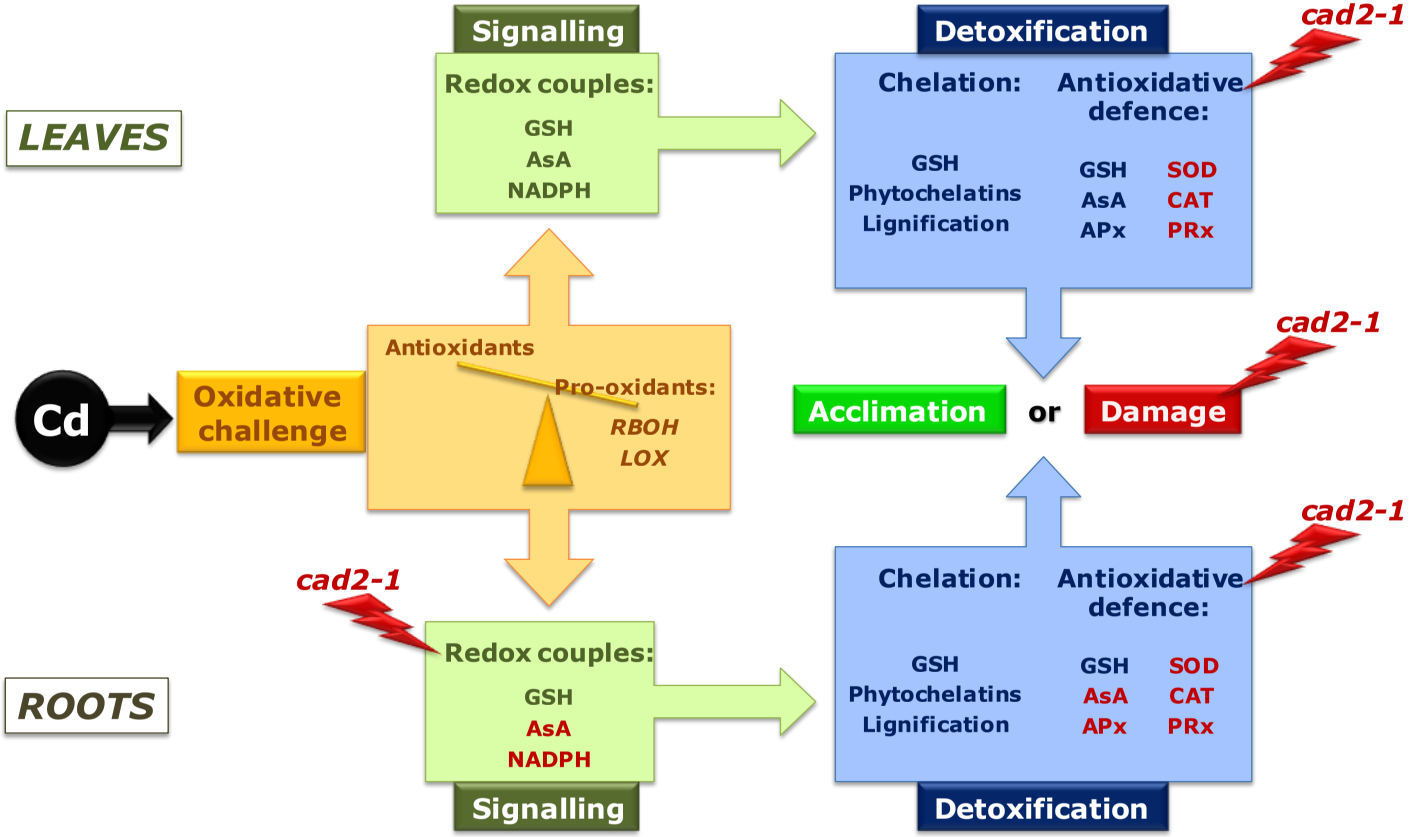


Fig. 4.9 *Schematic overview of root (lower part) and leaf (upper part) responses in wild-type Arabidopsis thaliana plants to cadmium (Cd) exposure. Glutathione (GSH) deficiency in the cad2-1 mutant resulted in the activation of additional antioxidants, which are indicated in a red font. Abbreviations: ascorbate (AsA), ascorbate peroxidase (APx), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), NADPH oxidase (RBOH) and peroxiredoxin (PRx).*

4.5.4 Conclusion

Deficiency in AsA influenced both growth and the cellular defence capacity under control conditions. The continuous priming caused the *vtc1-1* mutant to compensate its deficiency with elevated basal GSH levels and antioxidant transcripts. This might provide enhanced capacity for Cd chelation and ROS neutralisation and in its turn prepare the plants to deal with additional stresses like Cd toxicity. Glutathione deficiency however, was not associated with a distinctive phenotype or elevated basal defence mechanisms under control conditions. In addition, GSH deficient mutants were unable to compensate for their low metabolite contents, resulting in elevated Cd sensitivity complemented by the activation of multiple alternative mechanisms. Finally, this study included the double mutant, deficient in both GSH and AsA, which demonstrated that although both metabolites were addressed upon Cd exposure in leaves, GSH appeared to be the key agent in fast responses in roots.

Appendices

Table A.4.1 *List of primers used in the Reverse Transcription quantitative PCR (RTqPCR). Exon-Exon-junction (E-E-jn).*

Gene	TAIR: locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
APX1	*AT1G07890*	Ascorbate peroxidase1	FW: TGCCACAAGGATAGGTCTGG REV: CCTTCCTTCTCTCCGCTCAA	Exon 5	101 bp	96.3%
APX2	*AT3G09640*	Ascorbate peroxidase2	FW: TTGCTGTTGAGATCACTGGAGGA REV: TGAGGCAGACGACCTTCAGG	Exon 3	91 bp	90.9%
CAT1	*AT1G20630*	Catalase1	FW: AAGTGCTTCATCGGGAAGGA REV: CTTCAACAAAACGCTTCACGA	E5-E6-jn	103 bp	97.6%
CAT2	*AT4G35090*	Catalase2	FW: AACTCCTCCATGACCGTTGGA REV: TCCGTTCCCTGTCGAAATTG	Exon 2	76 bp	98.3%
CAT3	*AT1G20620*	Catalase3	FW: TCTCCAACAACATCTCTTCCCTCA REV: GTGAAATTAGCAACCTTCTCGATCA	Exon 2	91 bp	95.6%
CSD1	*AT1G08830*	CuZn superoxide dismutase1	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5	102 bp	93.8%
CSD2	*AT2G28190*	CuZn superoxide dismutase2	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6	101 bp	93.9%
DHAR1	*AT5G16710*	Dehydroascorbate reductase1	FW: CCAGATTCACTTCCTTTCGTCAA REV: TTACATCCTCTGTTTCCGCCC	Exon 6	91 bp	94.0%
DHAR2	*AT1G75270*	Dehydroascorbate reductase2	FW: ATCAGATGGGTCTTGTAGGGAAGC REV: GTGCTCCTGATGTTCTCGGC	Exon 2	91 bp	95.1%
DHAR3	*AT1G19550*	Dehydroascorbate reductase3	FW: AACTCTTTCCCCGGCGATAA REV: CTGAATTTGCCTCTGTTGGCTC	Exon 1	92 bp	95.2%
F-box	*AT5G15710*	F-box protein	FW: TTTCGGCTGAGAGGTTCGAGT REV: GATTCCAAGACGTAAAGCAGATCAA	Exon 1	63 bp	87.2%
FSD1	*AT4G25100*	Fe superoxide dismutase1	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4	101 bp	88.8%
GME	*AT5G28840*	Guanosine diphosphate-mannose epimerase	FW: GTTCACCGGCTCACGGAAAT REV: TGGGCTTCAGACCCGTTCTT	Exon 2	91 bp	93.8%
GMP	*AT2G39770*	Guanosine diphosphate-mannose pyrofosforylase	FW: TCTCAGTTTCCCAAAGCCCC REV: TTCATCAACTCCAACTGCCTTAAGA	Exon 1	91 bp	90.1%
GR1	*AT3G24170*	Glutathione reductase1	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15	101 bp	94.79%
GR2	*AT3G54660*	Glutathione reductase2	FW: GCCCAGATGGATGGAACAGAT REV: TAGGGTTGGAGAATGTTGGCG	Exon 5	91 bp	96.4%
GSH1	*AT4G23100*	γ-Glutamylcysteine synthetase	FW: CCCTGGTGAACTGCCTTCA REV: CATCAGCACCTCTCATCTCCA	Exon 5	101 bp	98.6%
GSH2	*AT5G27380*	Glutathione synthetase	FW: GGACTCGTCGTTGGTGACAA REV: TCTGGGAATGCAGTTGGTAGC	Exon 11	101 bp	92.6%

Table A.4.1 *Continued.*

Gene	TAIR:locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
LOX1	*AT1G55020*	Lipoxygenase1	FW: TTGGCTAAGGCTTTTGTCGG REV: GTGGCAATCACAAACGGTTC	Exon 6	101 bp	94.4%
LOX2	*AT3G45140*	Lipoxygenase2	FW: TTTGCTCGCCAGACACTTG REV: GGGATCACCATAAACGGCC	Exon 3	102 bp	86.7%
MDAR1	*AT3G52880*	Monodehydroascorbate reductase1	FW: TGCACCAACCCCAACTATCAC REV: CACAGCTCAGCCAAATGGAGAG	Exon 3	91 bp	93.6%
MDAR2	*AT5G03630*	Monodehydroascorbate reductase2	FW: CCCAATTCCAGCAGCAACAT REV: TCCATATGAGCGTCCTGCACT	Exon 7	91 bp	93.7%
MDAR3	*AT3G09940*	Monodehydroascorbate reductase3	FW: AAATGATTGCGAGTTCACCAGG REV: TACGTGATCATTGGAGGTGGTGT	Exon 8	91 bp	96.6%
MDAR4	*AT3G27820*	Monodehydroascorbate reductase4	FW: GGTTACGCAGCTCTCGAATTCA REV: AGGCCTCTCATACGGTGCAA	Exon 1	93 bp	97.6%
MDAR5	*AT1G63940.2*	Monodehydroascorbate reductase5	FW: TAGTAGAAAGCGGATCGCCTGA REV: GCGCTTGCGAGTTTAGCCTT	Exon 16	91 bp	95.4%
MIR172a[1]	*AT2G28056*	MicroRNA172a	Target: AGAAUCUUGAUGAUGCUGCAU	Lot Number: 0906007		
MIR398a[1]	*AT2G03445*	MicroRNA398a	Target: UGUGUUCUCAGGUCACCCCUU	Lot Number: 0905011		
MIR398b/c[1]	*AT5G14545*	MicroRNA398b/c	Target: UGUGUUCUCAGGUCACCCCUG	Lot Number: 0905005		
PCS1	*AT5G44070*	Phytochelatin synthase1	FW: TGCCAAGGAGCTGAAATCTT REV: ACCGTGCCTTCAGAGTCATC	Exon 2	91 bp	95.1%
RBOHA	*AT5G07390*	NADPH oxidase A	FW: CATTTCGCTAGGCCAAACTG REV: TTCACTAACCCAGCTGCTCCA	Exon 11	104 bp	96.4%
RBOHB	*AT1G09090*	NADPH oxidase B	FW: GATCGCGACAACCTCGGATA REV: CGGAAGATGGACTGTTATTTGATTG	Exon 4	91 bp	99.6%
RBOHC	*AT5G51060*	NADPH oxidase C	FW: TCACCAGAGACTGGCACAATAAA REV: GATGCTCGACCTGAATGCTC	Exon 6	101 bp	92.3%
RBOHD	*AT5G47910*	NADPH oxidase D	FW: AACTCTCCGCTGATTCCAACG REV: TGGTCAGCGAAGTCTTTAGATTCCT	Exon 1	96 bp	104.2%
RBOHE	*AT1G19230*	NADPH oxidase E	FW: GTGATGCAAGATCAACCCTGA REV: GCCTTGCAAAATGTGTTCTCA	Exon 13	105 bp	91.6%
RBOHF	*AT1G64060*	NADPH oxidase F	FW: GGTGTCATGAACGAAGTTGCA REV: AATGAGAGCAGAACGAGCATCA	Exon 11	99 bp	96.6%
SAND	*AT2G28390*	SAND family	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13	61 bp	107.8%
YLS8	*AT5G08290*	Thioredoxin-like protein	FW: TTACTGTTTCGGTTGTTCTCCATTT REV: CACTGAATCATGTTCGAAGCAAGT	Exon 2	61 bp	98.5%

[1] *MicroRNA primers were purchased from Applied Biosystems, their primer details were not revealed.*

Table A.4.2 *Reverse Transcription quantitative PCR (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines derived from Bustin et al. 2009.*

Sample/Template	
Source	*Arabidopsis thaliana* roots or shoots in a hydroponic culture
Method of preservation	harvest in liquid N_2 then stored at -80°C
Storage time	two weeks
Handling	frozen
Extraction method	columns: *mir*Vana miRNA Isolation Kit* (Ambion, Lennik, Belgium)
RNA: DNA-free	gDNA Wipeout Buffer and Quantitect Reverse Transcription Kit* (Qiagen, Venlo, the Netherlands) intron-spanning primers and verification of single peak on melt curves
Concentration	NanoDrop®: ND-1000 Spectrofotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Microfluidics: Bioanalyzer* (Agilent Technologies, Waldbronn, Duitsland) for a representative subset of the samples
Assay optimisation/validation	
Accession number	see Table A.4.1
Amplicon details	exon location & amplicon size: see Table A.4.1
Primer sequence	see Table A.4.1
In silico	Primer-BLAST
Empirical	primer concentration (300 nM) annealing temperature (60°C)
Priming conditions	oligo-dT and random primers
PCR efficiency	dilution curves (slope, y-intercept & r^2, see Table A.4.1)
Linear dynamic range	samples are within the efficiency curve
RT/PCR	
Protocols	Quantitect Reverse Transcription Kit* (Qiagen) Fast SYBR Green* (Applied Biosystems, Paisley, UK) see Materials and Methods
Reagents	see Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software, version 1.4 (Applied Biosystems, Lennik, Belgium, 2001-2006)
Statistical justification	5 biological replicates linear mixed-effects one-way ANOVA and Tukey post-hoc adjustment for multiple comparison
Normalisation	3 reference genes selected using geNorm, version 3.5 (Centre for Medical Genetics, Ghent, Belgium, 2001-7)

*All procedures were performed according to the manufacturer's protocol.

Table A.4.3: *Gene expression patterns relative to the wild-type control condition (1.00) in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) under control conditions in hydroponics; n = 5. Genes: γ-glutamylcysteine synthetase (GSH1), glutathione synthetase (GSH2), phytochelatin synthase (PCS1), GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose 3',5'-epimerase (GME), iron superoxide dismutase (FSD1), copper/zinc superoxide dismutase (CSD1/2), microRNA398 (MIR398a-b/c), catalase (CAT1-3), ascorbate peroxidase (APX1/2), monodehydroascorbate reductase (MDAR1-5), dehydroascorbate reductase (DHAR1-3), glutathione reductase (GR1/2), glutaredoxin (GRX13/480), peroxiredoxin (PRX-B/D), thioredoxin (ATTRX2 and ATHM3), NADPH oxidase (RBOHA-F), lipoxygenase (LOX1/2). Due to low abundance in the respective organ (*), these genes could not be analysed.*

ROOTS				GENES	LEAVES			
wild-type	***cad2-1***	***vtc1-1***	***cad2-1 vtc1-1***		***wild-type***	***cad2-1***	***vtc1-1***	***cad2-1 vtc1-1***
GSH/AsA biosynthesis								
1.00 ± 0.05	1.04 ± 0.02	0.97 ± 0.05	0.84 ± 0.20	***GSH1***	1.00 ± 0.01	1.02 ± 0.05	0.96 ± 0.04	0.82 ± 0.08
1.00 ± 0.10	1.21 ± 0.05	1.01 ± 0.11	0.79 ± 0.29	***GSH2***	1.00 ± 0.05	0.93 ± 0.09	0.85 ± 0.03	0.74 ± 0.10
1.00 ± 0.17	1.20 ± 0.05	1.16 ± 0.14	1.19 ± 0.17	***PCS1***	1.00 ± 0.17	0.85 ± 0.04	1.25 ± 0.22	1.43 ± 0.20
1.00 ± 0.02	1.52 ± 0.07	0.64 ± 0.09	0.52 ± 0.14	***GMP***	1.00 ± 0.04	1.08 ± 0.04	0.47 ± 0.04	0.50 ± 0.06
1.00 ± 0.03	1.03 ± 0.03	1.10 ± 0.03	1.10 ± 0.11	***GME***	1.00 ± 0.19	0.80 ± 0.04	1.02 ± 0.15	0.80 ± 0.07
$O_2^{\cdot-}$ scavenging								
1.00 ± 0.16	2.21 ± 0.29	1.44 ± 0.25	1.82 ± 0.44	***FSD1***	1.00 ± 0.36	1.45 ± 0.26	1.99 ± 0.38	2.47 ± 0.38
1.00 ± 0.07	1.02 ± 0.04	1.04 ± 0.03	0.67 ± 0.22	***CSD1***	1.00 ± 0.11	0.94 ± 0.03	0.75 ± 0.13	0.47 ± 0.08
1.00 ± 0.04	1.02 ± 0.03	1.10 ± 0.03	0.77 ± 0.21	***CSD2***	1.00 ± 0.12	0.90 ± 0.07	0.70 ± 0.14	0.36 ± 0.09
1.00 ± 0.13	0.70 ± 0.12	0.80 ± 0.08	1.24 ± 0.14	***MIR398a***	1.00 ± 0.30	1.30 ± 0.17	1.29 ± 0.31	0.99 ± 0.15
1.00 ± 0.16	1.78 ± 0.26	1.95 ± 0.21	3.27 ± 0.35	***MIR398b/c***	1.00 ± 0.33	1.41 ± 0.12	1.17 ± 0.29	1.27 ± 0.13
H_2O_2 scavenging/AsA-GSH cycle								
1.00 ± 0.09	0.90 ± 0.10	1.07 ± 0.13	0.64 ± 0.14	***CAT1***	1.00 ± 0.17	1.08 ± 0.22	0.93 ± 0.19	0.78 ± 0.07
1.00 ± 0.09	2.36 ± 0.26	2.30 ± 0.40	1.90 ± 0.46	***CAT2***	1.00 ± 0.22	0.86 ± 0.12	1.33 ± 0.27	1.02 ± 0.11
1.00 ± 0.28	3.16 ± 0.28	1.11 ± 0.24	1.18 ± 0.42	***CAT3***	1.00 ± 0.24	1.34 ± 0.29	1.01 ± 0.28	1.09 ± 0.25
1.00 ± 0.08	1.42 ± 0.08	1.29 ± 0.11	1.00 ± 0.32	***APX1***	1.00 ± 0.01	1.25 ± 0.11	1.24 ± 0.05	1.27 ± 0.03
1.00 ± 0.13	1.03 ± 0.09	1.70 ± 0.07	0.85 ± 0.04	***APX2***	1.00 ± 0.09	2.57 ± 0.61	1.05 ± 0.14	0.85 ± 0.12
1.00 ± 0.07	0.98 ± 0.07	1.05 ± 0.06	1.31 ± 0.13	***MDAR1***	1.00 ± 0.04	0.96 ± 0.04	0.93 ± 0.04	1.04 ± 0.15
1.00 ± 0.10	1.07 ± 0.02	1.03 ± 0.11	1.04 ± 0.26	***MDAR2***	1.00 ± 0.11	1.10 ± 0.03	0.77 ± 0.05	1.24 ± 0.15
1.00 ± 0.13	0.94 ± 0.05	1.12 ± 0.21	1.27 ± 0.44	***MDAR3***	1.00 ± 0.27	0.97 ± 0.40	6.29 ± 1.32	15.5 ± 3.92
1.00 ± 0.08	1.41 ± 0.07	1.30 ± 0.24	1.13 ± 0.39	***MDAR4***	1.00 ± 0.03	1.26 ± 0.07	1.23 ± 0.09	1.41 ± 0.19
1.00 ± 0.11	0.96 ± 0.03	1.32 ± 0.06	0.84 ± 0.26	***MDAR5***	1.00 ± 0.03	1.22 ± 0.08	1.19 ± 0.05	1.18 ± 0.10
1.00 ± 0.06	0.74 ± 0.04	0.79 ± 0.04	0.82 ± 0.07	***DHAR1***	1.00 ± 0.04	1.14 ± 0.01	1.21 ± 0.06	1.19 ± 0.08
1.00 ± 0.10	1.41 ± 0.05	1.91 ± 0.31	1.50 ± 0.42	***DHAR2***	1.00 ± 0.03	1.42 ± 0.12	1.56 ± 0.05	2.16 ± 0.20
1.00 ± 0.08	0.94 ± 0.03	0.74 ± 0.04	0.59 ± 0.18	***DHAR3***	1.00 ± 0.06	1.61 ± 0.16	1.28 ± 0.08	1.52 ± 0.26
1.00 ± 0.20	0.76 ± 0.11	1.14 ± 0.15	0.73 ± 0.26	***GR1***	1.00 ± 0.15	1.00 ± 0.10	1.13 ± 0.10	1.19 ± 0.05
1.00 ± 0.07	1.15 ± 0.02	1.20 ± 0.11	0.99 ± 0.28	***GR2***	1.00 ± 0.06	1.01 ± 0.05	1.04 ± 0.11	0.92 ± 0.11
1.00 ± 0.41	0.05 ± 0.01	0.14 ± 0.04	0.26 ± 0.08	***GRX13***	1.00 ± 0.26	0.66 ± 0.06	1.64 ± 0.46	3.30 ± 0.33
1.00 ± 0.26	1.88 ± 0.13	2.47 ± 0.52	0.97 ± 0.36	***GRX480***	1.00 ± 0.34	0.67 ± 0.25	7.12 ± 2.49	15.3 ± 2.85
1.00 ± 0.12	1.17 ± 0.05	0.88 ± 0.06	0.71 ± 0.19	***PRX-B***	1.00 ± 0.03	1.01 ± 0.07	0.97 ± 0.02	0.91 ± 0.13
1.00 ± 0.03	1.04 ± 0.02	1.06 ± 0.08	0.31 ± 0.05	***PRX-D***	1.00 ± 0.05	1.05 ± 0.08	1.22 ± 0.02	1.33 ± 0.06
1.00 ± 0.03	1.08 ± 0.03	1.11 ± 0.04	0.91 ± 0.14	***ATTRX2***	1.00 ± 0.04	1.03 ± 0.07	1.28 ± 0.11	1.53 ± 0.15
1.00 ± 0.04	1.13 ± 0.07	0.97 ± 0.08	0.92 ± 0.12	***ATHM3***	1.00 ± 0.03	0.93 ± 0.05	1.00 ± 0.05	1.04 ± 0.06
ROS production								
1.00 ± 0.07	0.85 ± 0.06	1.03 ± 0.06	0.73 ± 0.06	***RBOHA****				
1.00 ± 0.14	1.97 ± 0.05	1.14 ± 0.21	0.84 ± 0.26	***RBOHB****				
1.00 ± 0.10	1.16 ± 0.06	1.16 ± 0.16	0.80 ± 0.16	***RBOHC***	1.00 ± 0.26	0.19 ± 0.09	0.46 ± 0.12	0.45 ± 0.14
1.00 ± 0.25	1.26 ± 0.07	1.00 ± 0.28	1.14 ± 0.43	***RBOHD***	1.00 ± 0.07	0.82 ± 0.07	1.06 ± 0.06	0.98 ± 0.16
1.00 ± 0.09	1.15 ± 0.07	1.15 ± 0.12	0.93 ± 0.18	***RBOHE***	1.00 ± 0.16	0.84 ± 0.09	0.90 ± 0.08	1.33 ± 0.22
1.00 ± 0.10	1.06 ± 0.03	1.09 ± 0.10	1.01 ± 0.21	***RBOHF***	1.00 ± 0.09	1.01 ± 0.10	1.40 ± 0.16	1.39 ± 0.07
1.00 ± 0.14	1.34 ± 0.12	1.28 ± 0.10	0.98 ± 0.38	***LOX1***	1.00 ± 0.09	0.88 ± 0.05	1.08 ± 0.09	1.06 ± 0.14
				LOX2*	1.00 ± 0.29	1.92 ± 0.10	1.47 ± 0.33	1.70 ± 0.22

Chapter 5

Both content and redox state of glutathione and ascorbate influence *Arabidopsis'* sensitivity towards cadmium

Research article – in preparation for Annals of Botany

5.1 Abstract

Cadmium (Cd) is a non-essential metal that elicits oxidative stress. Plants respond to this Cd toxicity via increasing their chelating and antioxidative capacities. They predominantly chelate Cd via glutathione (GSH) and phytochelatins (PCs), while antioxidative responses are mainly based on the use and recycling of both GSH and ascorbate (AsA), complemented by superoxide dismutase (SOD) and catalase (CAT). In addition, both metabolites act as a substrate for the regeneration of other essential antioxidants, which neutralise and regulate reactive oxygen species (ROS). Together, these functions influence the cellular redox state of GSH and AsA, which includes both their concentration and reduction/oxidation ratio. In this study, both parameters concerning GSH and AsA redox state were taken into consideration in plants exposed to sublethal Cd concentrations. Deficiency in GSH (*cad2-1* mutant), AsA (*vtc1-1* mutant) and both metabolites (*cad2-1 vtc1-1* mutant) demonstrated changes in plant GSH homeostasis under control conditions, resulting in different Cd sensitivities. In comparison to wild-type plants, elevated basal thiol levels and enhanced PC synthesis upon Cd exposure efficiently compensated AsA deficiency in *vtc1-1* plants and contributed to decreased sensitivity towards Cd. Glutathione deficient (*cad2-1* and *cad2-1 vtc1-1*) mutants however, showed a more oxidised GSH redox state, resulting in initial oxidative stress and a higher sensitivity to Cd. In order to cope with the present Cd stress, GSH deficient mutants activated multiple alternative pathways.

5.2 Introduction

Cadmium (Cd) pollution is a worldwide environmental and health concern. Even low concentrations of Cd are associated with mortality (Nawrot *et al.* 2008; Maruzeni *et al.* 2014). Mainly areas with high industrial or agricultural activities contain elevated Cd levels in the soil, disturbing the entire ecosystem (Vangronsveld & Clijsters 1994; Chary *et al.* 2008). At the cellular level, Cd toxicity elicits oxidative stress in different organisms (Bertin & Averbeck 2006; Cuypers *et al.* 2009). In plants, recent studies focus on the Cd-induced oxidative challenge and the role of reactive oxygen species (ROS) in signalling processes, which regulate cellular and molecular responses in plants (Keunen *et al.* 2013a;

Smeets *et al.* 2013). Since ROS are continuously produced during normal cell metabolism, their basal levels should be tightly controlled. They are key players in regulating plant development and responses to environmental changes, including abiotic stress (Mittler *et al.* 2011; Suzuki *et al.* 2012). During metal stress, accumulation of ROS could evoke either severe oxidative damage or important oxidative signalling to activate defence mechanisms. Whether ROS will act as damaging or signalling factors depends on the balance between ROS production and scavenging (Gratão et al. 2005; Miller et al. 2008; Keunen et al. 2011a).

Gadjev and co-workers (2006) identified five gene transcripts that were up-regulated more than fivefold upon several stress conditions resulting in oxidative stress: a gene up-regulated by oxidative stress (*UPOX*, *AT2G21640*), a defensin-like gene (*defensin-like*, *AT2G43510*), two genes with unknown function (*AT1G19020* and *AT1G05340*), and a gene of the Toll-Interleukin-1 class (*TIR*, *AT1G57630*). These genes are considered hallmark genes for the general oxidative stress response (Mittler *et al.* 2004; Gadjev *et al.* 2006). In the case of Cd exposure, plant's major cellular defences related to prevent accumulation of free metal ions and to neutralise excessive ROS levels are activated. Glutathione (GSH) is the most ubiquitous and abundant non-protein thiol in plant cells, containing a free thiol group on cysteine. Due to Cd's high affinity to thiols, polymerisation of GSH molecules into phytochelatins (PCs) is a fast response to Cd toxicity. Thiols are able to undergo redox reactions, providing GSH with both chelating and antioxidant properties (Brunetti *et al.* 2011; Jozefczak *et al.* 2012; Zagorchev *et al.* 2013; Jozefczak *et al.* 2014).

In plants, antioxidative responses are largely based on the use and recycling of both GSH and ascorbate (AsA), complemented by superoxide dismutase (SOD) and catalase (CAT). The SODs constitute the first line of defence against superoxide ($O_2^{\bullet -}$), the first ROS intermediate. The enzymes are classified into three groups based on their metal co-factor: iron (FeSOD), copper/zinc (CuZnSOD) and manganese SOD (MnSOD). These redox-active metals dismutate $O_2^{\bullet -}$ and produce the relatively stable hydrogen peroxide (H_2O_2). Both CAT and ascorbate peroxidase (APx) are major H_2O_2 scavengers preventing the formation of toxic hydroxyl radicals ($^{\bullet}OH$) (Mittler *et al.* 2004; Halliwell 2006). In contrast to APx, CAT has a low affinity for H_2O_2 but a high reaction speed and no

limitation by its substrate. These different characteristics make APx a good candidate for ROS fine tuning, while CAT might be responsible to remove the excess ROS (Cuypers *et al.* 2011). As stated before, GSH has primary antioxidant functions but it also acts as a substrate for the regeneration of other essential antioxidants like glutaredoxins and AsA. In the AsA-GSH cycle, both metabolites are successively oxidised and reduced, allowing APx to neutralise H_2O_2. Oxidised forms of GSH [*i.e.* glutathione disulfide (GSSG)] and AsA [*i.e.* dehydroascorbate (DHA)] are reduced back by NADPH-dependent glutathione reductase (GR) and GSH-dependent dehydroascorbate reductase (DHAR), respectively (Mittler *et al.* 2004; Jozefczak *et al.* 2012). Apart from its function as a substrate in the AsA-GSH cycle, AsA also directly neutralises ROS non-enzymatically and it is involved in the regeneration of α-tocopherol, which scavenges both ROS and lipid peroxyl radicals, and zeaxanthin, which is involved in the photoprotective xanthophyll cycle (Bielen *et al.* 2013).

In order to maintain protein structure and function, a reducing intracellular environment is essential. Both GSH/GSSG and AsA/DHA are major cellular redox buffers that keep the environment reduced. Changes in these ratios reflect the cellular toxicity and have been associated with redox signalling (Foyer & Noctor 2005a; Jozefczak *et al.* 2012; Zagorchev *et al.* 2013). The exact mechanism of this signalling system is still under intense investigation. Therefore, in the present study, both the concentration and redox state of GSH and AsA were investigated in roots and leaves of Cd-exposed *Arabidopsis thaliana* plants and related to downstream molecular responses. To increase the resolution of our knowledge concerning GSH- and AsA-related pathways, Cd-induced responses were compared between wild-type and mutant plants deficient in either one or both metabolites.

5.3 Materials and methods

5.3.1 Plant material, growth conditions and harvest

Wild-type (Lehle seeds, Round Rock TX, USA) and mutant *Arabidopsis thaliana* (cv. Columbia) seeds were surface sterilised and grown in a hydroponic culture [Hoagland solution; 12 h/21°C day, 12 h/17°C night regime; 170 µmol m^{-2} s^{-1} light at the leaf level delivered by cool white fluorescent lamps (L 140W/20SA,

Osram, Augsburg); and 65% relative humidity; Chapter 3]. The *cad2-1* mutant was donated by Dr. Christopher Cobbett (Melbourne University, Australia). This mutant was selected in a screen for Cd sensitivity and carries a mutation in the GSH biosynthesis gene *γ*-glutamylcysteine synthetase (*GSH1*, *AT4G23100*), resulting in approximately 30% of wild-type GSH levels (Cobbett *et al.* 1998). The *vtc1-1* mutant was obtained from the European *Arabidopsis* Stock Centre (uNASC ID: N8326, UK). This mutant was selected in a screen for vitamin C deficiency and carries a mutation in the AsA biosynthesis gene GDP-D-mannose pyrophosphorylase (*GMP*, *AT2G39770*), resulting in approximately 40-50% of wild-type AsA levels (Conklin *et al.* 1999). The *cad2-1 vtc1-1* double mutant was donated by Dr. Mark Aarts (Wageningen University, the Netherlands; Clerkx *et al.* 2004). After a period of three weeks growth, plants were exposed to 0, 1 or 5 µM Cd supplied as $CdSO_4$ in the Hoagland solution. After 72 h of exposure, the aerial part and roots were separated and dried prior to element determination or snap frozen in liquid nitrogen, then stored at -80°C, prior to the other measurements.

5.3.2 Element determination

At harvesting, roots were rinsed with distilled water and incubated for 30 min in an ice-cold desorbing solution of 5 mM $PbNO_3$. Subsequently, roots were washed three times in distilled water and once in Millipore water. Leaves were rinsed three times with Millipore water. The fresh plant material was dried at 60°C, weighed, and digested in 70-71% HNO_3 using a heat block. Element concentrations were measured via inductively coupled plasma-optical emission spectrometry (ICP-OES 710, Agilent Technologies, Australia).

5.3.3 Metabolite measurements

Analysis of AsA, GSH and PC contents were performed with reverse-phase HPLC as previously described (Semane *et al.* 2007). For total AsA and GSH measurements, frozen tissues were ground in liquid nitrogen and homogenised in 6% (w/v) meta-phosphoric acid. Samples were centrifuged (15 min, 14 000 *g*, 4°C) and the supernatant was incubated with dithiothreitol. After 15 min this reduction was stopped by adding acetonitrile and the samples were separated at 40°C on a reverse-phase C_{18} column (type Polaris 3 C_{18}, 3 µm, 100 x 4.6 mm, Varian, the Netherlands) with an isocratic flow of 0.8 ml min^{-1} of the elution

buffer (25 mM KPO_4, pH 3). Detection of AsA and GSH occurred between 190-250 nm via diode array (SPDM10AVP, Shimadzu, The Netherlands), set in tandem with a homemade amperometric detection system (glassy carbon working electrode, calomel reference electrode, reference potential 1000 mV). For PC analysis, samples were ground in 6.3 mM diethylenetriaminepentaacetic acid with 0.1% (v/v) trifluoroacetic acid at 4°C supplemented with 10mM N-acetyl cysteine as an internal standard. The samples were filtered and the thiols were derivatised with 25 mM monobromobimane (30 min, 45°C). The thiols were separated at 37°C on two tandemly arranged Nova-Pak C_{18} columns (6 nm, 4 µm, 3.9 x 150 mm, Waters, Milford, MA), using a slightly concave gradient of 12-25% (v/v) methanol (15 min) and then a linear gradient from 25-50% (v/v) methanol (15-40 min). Fluorescence was monitored using a Waters 474 fluorescence detector. Quantification was based on the internal standard and GSH standards.

5.3.4 RNA extraction, reverse transcription and gene expression

Samples were disrupted under frozen conditions using two stainless steel beads and the Retch Mixer Mill MM 2000 (Retsch, Germany). RNA was extracted using the RNAqueous Total RNA Isolation Kit (Ambion, Belgium). The TURBO DNA-free kit (Ambion) and the High Capacity cDNA Reverse Transcription Kit (Ambion, random hexamer primers and 1 µg RNA input) were used to remove genomic DNA and to carry out reverse transcription, respectively. A tenfold dilution of the cDNA in 1/10 diluted TE-buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) was stored at -20°C. Quantitative PCR (qPCR) was performed using Fast SYBR Green chemistry according to the manufacturer's instructions on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Belgium). Relative gene expression was calculated as $2^{-\Delta Cq}$ and was normalised with a normalisation factor based on the expression of the following reference genes: *AT5G55840*, *AT2G28390* and *AT4G34270* for roots and *AT2G28390*, *AT5G25760* and *AT4G34270* for leaves (Remans *et al.* 2008). Gene-specific primers (300 nM, Table A.5.1 in Appendices) were designed and optimised using Primer Express (Applied Biosystems). Table A.5.1 in Appendices shows the Reverse Transcription quantitative (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines (Bustin *et al.* 2009).

5.3.5 Clustering of gene expression data

Hierarchical clustering analysis was performed (GenEx software, v6, MultiD Analyses AB, Sweden) to recognise potential sample-related patterns during Cd exposure in four *Arabidopsis thaliana* genotypes (wild-type, *cad2-1*, *vtc1-1* and *cad2-1 vtc1-1*). The analysis was based on raw gene expression values and the "Average linkage" algorithm, defining the distance between conditions as the average of distances between all pairs of individuals in all groups. Distances between the measures were calculated via the Euclidian Distance Measure. Heat maps were constructed to compare expression levels between different genes and samples.

5.3.6 Statistical analysis

All data were analysed with general linear ANOVA models (Verbeke & Molenberghs 2000). Normal distribution and homoscedasticity were tested using the Shapiro-Wilk and Bartlett test, respectively. Tukey post-hoc adjustment was used to correct for multiple comparison. Logarithmic transformations were applied when necessary to approximate normality, gene expression data were always log-transformed. All statistical analyses were performed using R (the R foundation of statistical computing, version 2.15.1). Data are mean values ± standard error (SE) and significance was set at the 5% level ($P < 0.05$).

5.4 Results

5.4.1 Phenotypic characteristics of Arabidopsis thaliana genotypes: wild-type, glutathione-deficient, ascorbate-deficient and double mutant plants

After three weeks of growth under control conditions, wild-type and GSH deficient plants (*cad2-1*) showed a similar phenotype, while both AsA deficient mutants (*vtc1-1* and *cad2-1 vtc1-1*) differed from the wild-type plants. First, their rosettes illustrated a retarded growth and yellowing of the cotyledons (Fig. 5.1). Secondly, their root and leaf fresh weight was reduced (Table 5.1). Although wild-type fresh weight only revealed decreasing trends after exposure to Cd, a significant decrease was observed in roots and leaves of *cad2-1* plants. The *vtc1-1* mutant however, did not show a reduced growth after Cd exposure but its cotyledons turned more yellow. The double mutant did show a significantly reduced root fresh weight and its rosette appeared small and with necrotic lesions in comparison to its control condition.

Table 5.1 *Fresh weight (mg FW plant^{-1}) of roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0, 1 or 5 µM $CdSO_4$ for 72 h in hydroponics. Statistical significance is expressed using lower (roots) and upper case letters (leaves); n = 5 (two-way ANOVA, P < 0.05).*

Fresh weight (mg plant^{-1})	0 µM Cd	1 µM Cd	5 µM Cd
	LEAVES		
wild-type	95.75 ± 4.52^{A}	73.80 ± 7.42^{AB}	74.60 ± 5.35^{AB}
cad2-1	119.60 ± 14.89^{A}	80.60 ± 8.49^{AB}	41.10 ± 4.34^{BC}
vtc1-1	44.00 ± 1.78^{BC}	43.00 ± 4.66^{BC}	48.17 ± 10.91^{BC}
cad2-1 vtc1-1	32.75 ± 2.15^{C}	24.48 ± 4.05^{C}	28.21 ± 4.37^{C}
	ROOTS		
wild-type	70.00 ± 2.68^{a}	42.70 ± 4.87^{ab}	37.40 ± 3.45^{ab}
cad2-1	67.30 ± 8.98^{a}	36.90 ± 6.29^{ab}	12.57 ± 1.48^{bc}
vtc1-1	17.28 ± 1.89^{bc}	8.26 ± 1.40^{bc}	21.98 ± 7.61^{bc}
cad2-1 vtc1-1	9.14 ± 1.27^{c}	7.24 ± 2.28^{cd}	3.05 ± 0.57^{d}

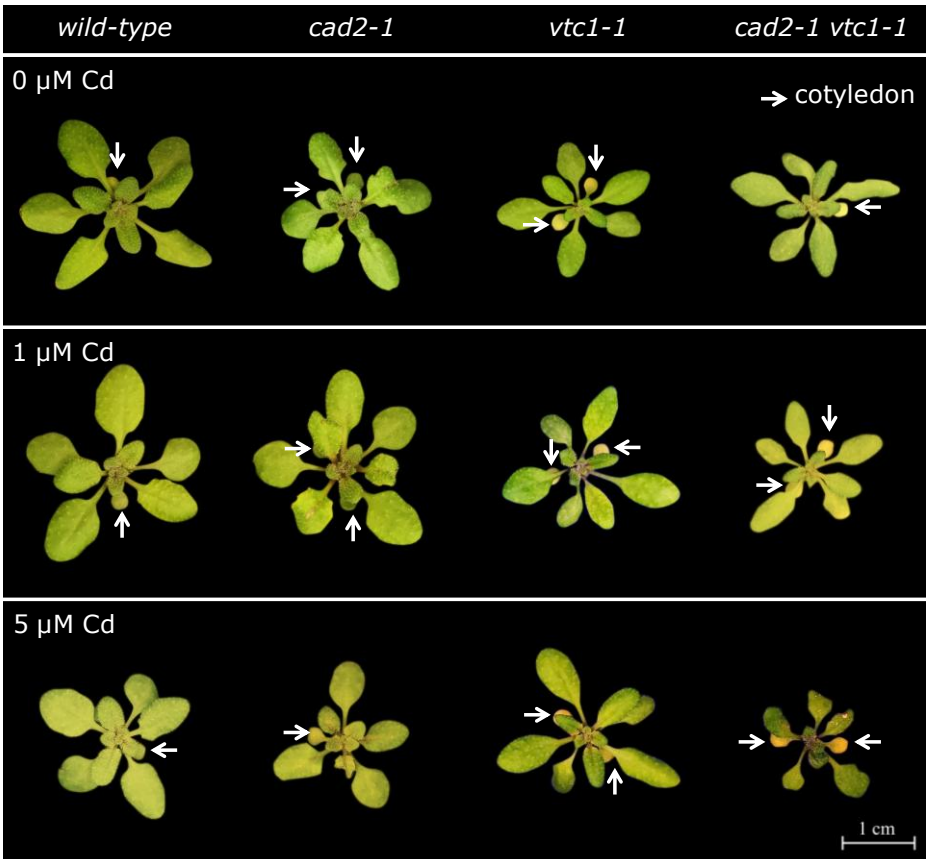


Fig. 5.1 *Representative pictures of the rosette appearance of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0, 1 or 5 µM $CdSO_4$ for 72 h in hydroponics; n = 28. Cotyledons are indicated by a white arrow.*

5.4.2 Genotype-specific accumulation of elements

Exposure to Cd raised Cd levels in a dose-dependent manner in both organs of all genotypes (Fig. 5.2A). However, the mutants showed increasing trends in root Cd levels and decreasing trends in the leaves. This resulted in lower Cd translocation factors in the mutants, which were significant in the double mutant (Fig. 5.2B). Copper levels were similar in all genotypes, except the double mutant contained an increased Cu content in its roots under control conditions (Fig. 5.2C). After exposure to Cd, a dose-dependent increase in Cu levels was detected in roots, while leaf Cu levels were constant. Concerning K, equal levels were detected in all genotypes under control conditions (Fig. 5.2D). Exposure to Cd however, resulted in a decrease of K levels in roots of both GSH deficient mutants (*cad2-1* and *cad2-1 vtc1-1*).

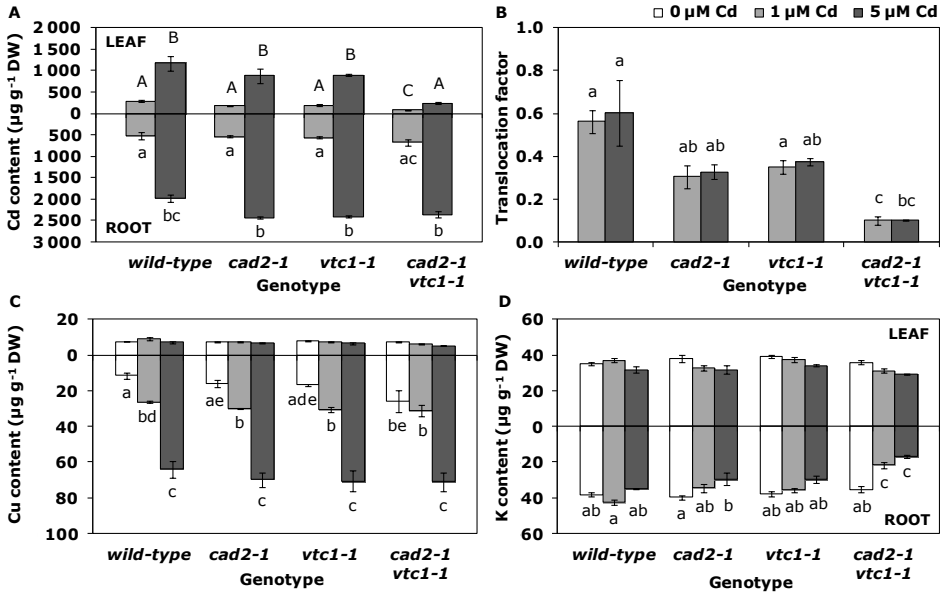


Fig. 5.2 *(A, C, D) Root and leaf element concentrations (µg g^{-1} DW) and (B) the corresponding translocation factor of Cd from roots to shoots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 (□), 1 (▒) or 5 (■) µM $CdSO_4$ for 72 h in hydroponics. A, cadmium (Cd) content; C, copper (Cu) content; and D, potassium (K) content. Statistical significance is expressed using lower (roots and translocation factor) and upper case letters (leaves); n = 4 (two-way ANOVA, P < 0.05).*

5.4.3 Altered metabolite levels in Arabidopsis genotypes

Under control conditions, the mutant genotypes were confirmed: in comparison to wild-type plants, *vtc1-1* mutants contained 45-65% AsA (Fig. 5.3), *cad2-1* mutants contained approximately 30% GSH (Fig. 5.4), and *cad2-1 vtc1-1* mutants contained 40-50% AsA and approximately 30% GSH. Exposure to Cd resulted in a dose-dependent decrease in total AsA levels in the roots of all genotypes, while wild-type and *cad2-1* leaf AsA levels increased dose-dependently.

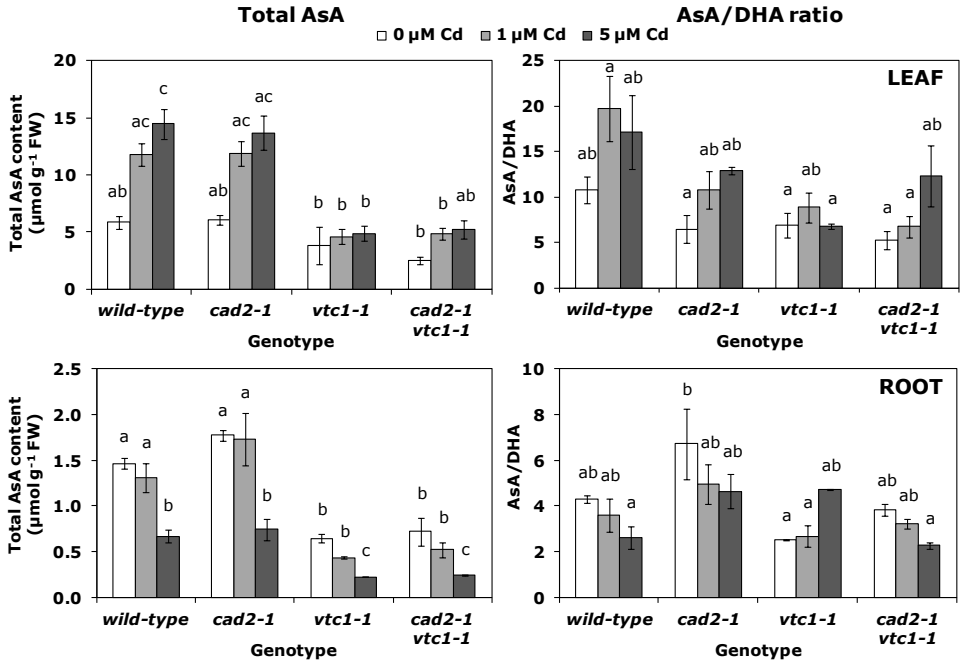


Fig. 5.3 *Concentration of total ascorbate (AsA) and the ratio between AsA and dehydroascorbate (DHA) in leaves and roots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 (□), 1 (▒) or 5 (■) µM $CdSO_4$ for 72 h in hydroponics. Statistical significance is expressed using lower case letters; n = 4 (two-way ANOVA, P < 0.05).*

The GSH/GSSG ratio under control conditions was significantly lower in both GSH deficient mutants in comparison to the other genotypes. While Cd exposure resulted in a significantly lower GSH/GSSG ratio (*i.e.* a more oxidised GSH redox state) in roots of wild-type and *cad2-1* plants, both AsA deficient mutants showed decreasing trends. In leaves however, a general reduction of the GSH redox state was apparent, although only significant in leaves of *cad2-1* plants.

Phytochelatins were absent under control conditions in roots, except in *vtc1-1* mutants a low concentration of PC2 (*i.e.* 14.75 nmol GSH equivalents g^{-1} FW) was detected (Fig. 5.5). After exposure to Cd, a dose-dependent increase in root PCs was detected in all genotypes. In the roots of *vtc1-1* plants, PC levels were approximately twofold higher than in wild-type plants under the same condition. In both GSH deficient plants however, no PCs were detected after exposure to 1 µM Cd and less than 15% of wild-type PC levels were present after exposure to 5 µM Cd.

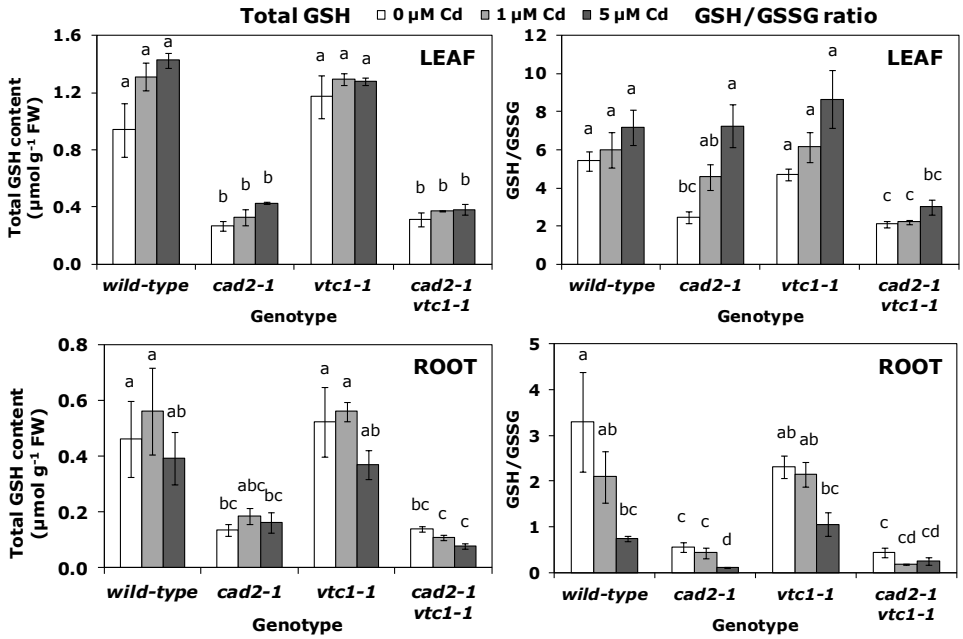


Fig. 5.4 *Concentration of total glutathione (GSH) and the ratio between GSH and glutathione disulfide (GSSG) in leaves and roots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 (□), 1 (▩) or 5 (■) µM $CdSO_4$ for 72 h in hydroponics. Statistical significance is expressed using lower case letters; n = 4 (two-way ANOVA, P < 0.05).*

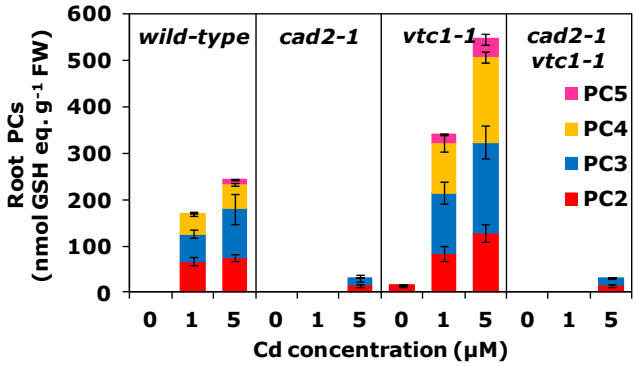


Fig. 5.5 *Profiling the phytochelatin (PC) content [PC2 (■), PC3 (■), PC4 (■) and PC5 (■)] in four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) in roots exposed to 0, 1 or 5 µM $CdSO_4$ for 72 h in hydroponics. Thiol content is expressed in GSH equivalents (nmol g^{-1} FW).*

5.4.4 Glutathione and ascorbate deficient plants possess a different gene expression profile

In roots, only the GSH deficient mutants significantly induced glutathione synthetase (*GSH2*) expression after exposure to 5 µM Cd (Table 5.2). However, all genotypes activated the SOD pathway in a dose-dependent manner, *i.e.* increasing FeSOD (*FSD1*) and microRNA398 [primary microRNA398 transcripts (*pri-MIR398a-c*)] expression, resulting in decreasing CuZnSOD (*CSD1/2*) transcript levels. Concerning *CAT* genes, both *CAT1* and *CAT2* were down-regulated in all conditions, except in the GSH deficient mutants, where *CAT1* was significantly up-regulated. Although *APX1* was down-regulated, *APX2* was strongly induced in all genotypes except in the roots of *vtc1-1* plants. No significant changes were detected in *DHAR* expression except in the double mutant, which showed decreased *DHAR2* and increased *DHAR3* transcript levels. Together with the roots of *cad2-1* plants, the double mutant possessed increased levels of *GR1* transcripts. Finally, the oxidative stress marker genes were induced in all genotypes in a dose-dependent manner. On the one hand, in the roots of wild-type and *vtc1-1* plants, three out of five investigated genes were up-regulated (more than fivefold) to the same extent. On the other hand, both GSH deficient mutants induced all genes and often to a significantly greater extent than in roots of wild-type plants. Additionally, these mutants possessed elevated transcript levels of *UPOX* and the *defensin-like* gene in both organs under control conditions (Fig. 5.6). Taken the gene expression data together in a heat map, hierarchical agglomerate clustering revealed two clusters in roots exposed to 5 µM Cd. One cluster contained wild-type and *vtc1-1* plants showing mostly down-regulated expressions, and the second cluster included both GSH deficient mutants of which the roots of *cad2-1* plants displayed more up-regulated genes and the double mutant showed a combination of both colours evenly distributed (Fig. 5.7A).

In leaves, only the *cad2-1* mutant induced *GSH2* expression (Table 5.2). The SOD pathway was activated but to a lesser extent than in roots: *CSD1/2* transcripts were decreased and *pri-MIR398a-c* was significantly up-regulated in some conditions but no significant induction of *FSD1* was present. Concerning leaf *DHAR* levels, *cad2-1* plants showed a significant down-regulation of *DHAR1* and a significant up-regulation of *DHAR2-3*. Also the double mutant showed

increased *DHAR2-3* transcript levels. In addition, the leaves of *cad2-1* plants demonstrated an increased *GR1* and decreased *GR2* expression after exposure to the highest Cd concentration. In leaves of both wild-type and *vtc1-1* plants exposed to 5 µM Cd, the expression level of one oxidative stress marker gene was significantly induced but only to a minor extent. In the double mutant, no significant changes were detected, while the leaves of *cad2-1* plants showed more than fivefold increased expression levels of three oxidative stress marker genes. Finally, an interesting genotype-effect was detected in the leaves of *cad2-1* plants under control conditions: *APX2* transcripts were more than tenfold increased in comparison to wild-type plants (Fig. 5.8). The heat map for leaves exposed to 5 µM Cd demonstrates that the *cad2-1* mutant clustered apart from the other genotypes (Fig. 5.7B).

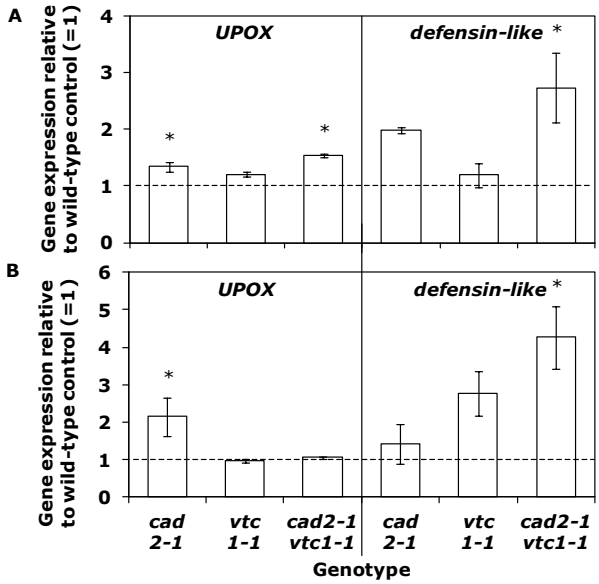


Fig. 5.6 *Gene expression of a gene up-regulated by oxidative stress (UPOX) and a defensin-like gene in roots (A) and leaves (B) of three Arabidopsis thaliana mutants (cad2-1, vtc1-1 and cad2-1 vtc1-1) under control conditions. Gene expression is relatively expressed to wild-type control (1: dashed line, n = 4, two-way ANOVA, significance level: * P < 0.05).*

Table 5.2 *Gene expression patterns relative to the control of the corresponding genotype (=1.00) in roots and leaves of four Arabidopsis thaliana genotypes: wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1. Plants were exposed to 1 or 5 µM $CdSO_4$ for 72 h in hydroponics. Significant treatment effects relative to the control of the corresponding genotype (up-regulation, ■; down-regulation, ■) and relative to the same condition in wild-type (up-regulation, ■; down-regulation, ■; two-way ANOVA; P < 0.05; n = 4). Genes: γ-glutamylcysteine synthetase (GSH1), glutathione synthetase (GSH2), phytochelatin synthase (PCS1), iron superoxide dismutase (FSD1), copper/zinc superoxide dismutase (CSD1/2), primary microRNA398 transcripts (pri-MIR398a-b/c), catalase (CAT1-3), ascorbate peroxidase (APX1/2), dehydroascorbate reductase (DHAR1-3), glutathione reductase (GR1/2), Toll-Interleukin-1 class (TIR), up-regulated by oxidative stress (UPOX).*

72 h	ROOTS				Genes	LEAVES				72 h
	wild-type	*cad2-1*	*vtc1-1*	*cad2-1 vtc1-1*		*wild-type*	*cad2-1*	*vtc1-1*	*cad2-1 vtc1-1*	
Cd	**GSH biosynthesis**									Cd
1 µM	0.82 ± 0.04	0.96 ± 0.05	0.93 ± 0.05	0.99 ± 0.03	***GSH1***	1.07 ± 0.02	1.10 ± 0.08	1.02 ± 0.04	1.01 ± 0.02	1 µM
5 µM	0.82 ± 0.02	1.17 ± 0.05	0.94 ± 0.05	1.14 ± 0.10		1.02 ± 0.01	1.08 ± 0.12	0.91 ± 0.07	1.13 ± 0.03	5 µM
1 µM	0.97 ± 0.06	1.23 ± 0.05	1.14 ± 0.06	1.22 ± 0.09	***GSH2***	1.08 ± 0.12	1.72 ± 0.18	0.94 ± 0.03	1.00 ± 0.02	1 µM
5 µM	1.05 ± 0.09	2.01 ± 0.11	1.09 ± 0.01	1.43 ± 0.22		0.96 ± 0.05	1.70 ± 0.24	0.88 ± 0.03	1.18 ± 0.03	5 µM
1 µM	1.02 ± 0.07	1.22 ± 0.09	0.96 ± 0.05	0.89 ± 0.08	***PCS1***	0.62 ± 0.13	0.98 ± 0.04	1.06 ± 0.12	0.99 ± 0.05	1 µM
5 µM	1.27 ± 0.18	1.12 ± 0.10	1.41 ± 0.08	1.05 ± 0.03		0.79 ± 0.09	0.99 ± 0.03	1.23 ± 0.06	0.90 ± 0.03	5 µM
	$O_2^{\bullet-}$-scavenging									
1 µM	2.18 ± 0.27	3.87 ± 0.51	1.82 ± 0.23	2.23 ± 0.10	***FSD1***	0.91 ± 0.41	1.79 ± 0.45	0.89 ± 0.16	1.07 ± 0.21	1 µM
5 µM	9.70 ± 1.16	6.23 ± 0.84	7.76 ± 1.91	9.05 ± 0.88		1.91 ± 0.29	1.46 ± 0.35	0.75 ± 0.10	1.42 ± 0.03	5 µM
1 µM	0.83 ± 0.02	0.77 ± 0.04	1.09 ± 0.07	1.09 ± 0.02	***CSD1***	0.60 ± 0.05	0.75 ± 0.06	0.51 ± 0.17	0.65 ± 0.07	1 µM
5 µM	0.40 ± 0.02	0.56 ± 0.05	0.52 ± 0.02	0.45 ± 0.06		0.31 ± 0.06	0.46 ± 0.08	0.51 ± 0.07	0.35 ± 0.04	5 µM
1 µM	0.75 ± 0.03	0.70 ± 0.04	1.01 ± 0.07	1.06 ± 0.06	***CSD2***	0.73 ± 0.13	0.35 ± 0.02	0.42 ± 0.14	0.66 ± 0.09	1 µM
5 µM	0.50 ± 0.00	0.56 ± 0.03	0.60 ± 0.02	0.43 ± 0.03		0.22 ± 0.05	0.11 ± 0.02	0.44 ± 0.08	0.25 ± 0.02	5 µM
1 µM	4.35 ± 0.63	6.60 ± 1.01	5.33 ± 0.82	3.03 ± 0.87	***pri-MIR398a***	1.47 ± 0.35	2.01 ± 0.28	2.70 ± 1.20	2.09 ± 0.57	1 µM
5 µM	8.70 ± 2.59	27.4 ± 4.02	13.7 ± 2.36	10.9 ± 2.06		15.6 ± 10.21	2.70 ± 0.28	6.25 ± 0.72	5.42 ± 1.53	5 µM
1 µM	1.17 ± 0.11	2.28 ± 0.13	1.28 ± 0.12	1.66 ± 0.10	***pri-MIR398b***	0.59 ± 0.16	1.79 ± 0.13	1.51 ± 0.38	1.20 ± 0.16	1 µM
5 µM	2.54 ± 0.39	3.92 ± 0.72	2.10 ± 0.23	4.54 ± 0.52		2.23 ± 0.06	2.52 ± 0.18	0.99 ± 0.12	2.01 ± 0.24	5 µM
1 µM	3.26 ± 0.97	16.1 ± 1.84	2.82 ± 0.75	5.02 ± 0.95	***pri-MIR398c***	0.32 ± 0.10	3.19 ± 0.36	2.35 ± 0.75	1.78 ± 0.29	1 µM
5 µM	18.2 ± 5.47	34.7 ± 5.16	7.53 ± 1.20	27.6 ± 4.95		2.10 ± 0.12	5.91 ± 0.88	1.37 ± 0.25	2.83 ± 0.23	5 µM
	H_2O_2-scavenging/AsA-GSH cycle									
1 µM	0.73 ± 0.01	1.48 ± 0.05	0.87 ± 0.04	1.34 ± 0.03	***CAT1***	0.77 ± 0.08	1.00 ± 0.08	0.73 ± 0.09	0.78 ± 0.11	1 µM
5 µM	0.69 ± 0.03	2.49 ± 0.34	0.73 ± 0.05	2.17 ± 0.13		0.67 ± 0.04	1.57 ± 0.22	0.70 ± 0.07	0.95 ± 0.08	5 µM
1 µM	0.70 ± 0.03	0.75 ± 0.05	0.73 ± 0.04	0.72 ± 0.06	***CAT2***	0.92 ± 0.06	0.60 ± 0.02	0.92 ± 0.04	1.01 ± 0.06	1 µM
5 µM	0.64 ± 0.03	0.58 ± 0.07	0.72 ± 0.05	0.47 ± 0.01		0.81 ± 0.02	0.40 ± 0.01	0.81 ± 0.04	0.83 ± 0.04	5 µM
1 µM	0.69 ± 0.06	0.92 ± 0.09	0.70 ± 0.07	0.91 ± 0.15	***CAT3***	0.84 ± 0.13	1.17 ± 0.11	0.71 ± 0.10	1.14 ± 0.27	1 µM
5 µM	0.65 ± 0.02	1.71 ± 0.12	0.84 ± 0.09	1.80 ± 0.40		1.37 ± 0.26	1.43 ± 0.19	0.69 ± 0.07	1.68 ± 0.07	5 µM
1 µM	0.85 ± 0.03	0.87 ± 0.06	0.95 ± 0.03	0.93 ± 0.01	***APX1***	0.75 ± 0.02	0.76 ± 0.06	0.86 ± 0.14	0.96 ± 0.11	1 µM
5 µM	0.79 ± 0.03	0.76 ± 0.03	0.95 ± 0.02	0.85 ± 0.09		0.77 ± 0.09	0.78 ± 0.10	0.71 ± 0.05	0.98 ± 0.06	5 µM
1 µM	3.15 ± 0.48	1.84 ± 0.38	1.35 ± 0.18	3.28 ± 0.79	***APX2***	0.79 ± 0.12	0.17 ± 0.04	2.13 ± 0.43	1.33 ± 0.37	1 µM
5 µM	1.21 ± 0.35	4.78 ± 0.60	0.95 ± 0.06	5.84 ± 0.94		1.72 ± 0.59	1.88 ± 0.15	1.78 ± 0.11	2.46 ± 0.93	5 µM
1 µM	0.87 ± 0.03	0.95 ± 0.04	1.00 ± 0.06	1.05 ± 0.03	***DHAR1***	0.93 ± 0.05	0.71 ± 0.03	0.95 ± 0.06	1.06 ± 0.07	1 µM
5 µM	0.85 ± 0.07	0.94 ± 0.07	0.85 ± 0.03	1.02 ± 0.03		0.78 ± 0.03	0.44 ± 0.04	0.80 ± 0.02	0.87 ± 0.03	5 µM
1 µM	0.97 ± 0.10	0.99 ± 0.04	1.12 ± 0.03	0.90 ± 0.06	***DHAR2***	1.06 ± 0.06	1.42 ± 0.11	1.13 ± 0.08	1.13 ± 0.06	1 µM
5 µM	1.03 ± 0.11	0.82 ± 0.02	1.24 ± 0.07	0.71 ± 0.05		1.30 ± 0.08	1.48 ± 0.10	1.00 ± 0.06	1.41 ± 0.06	5 µM
1 µM	0.68 ± 0.02	0.82 ± 0.01	1.22 ± 0.05	1.28 ± 0.10	***DHAR3***	1.61 ± 0.23	1.22 ± 0.08	1.30 ± 0.16	1.41 ± 0.25	1 µM
5 µM	0.84 ± 0.05	0.74 ± 0.03	1.17 ± 0.07	2.26 ± 0.24		1.93 ± 0.37	2.12 ± 0.26	0.80 ± 0.09	2.18 ± 0.05	5 µM
1 µM	1.02 ± 0.07	1.25 ± 0.04	1.25 ± 0.03	1.34 ± 0.05	***GR1***	0.89 ± 0.02	1.18 ± 0.05	1.00 ± 0.02	0.92 ± 0.03	1 µM
5 µM	1.20 ± 0.09	1.86 ± 0.02	1.23 ± 0.06	1.70 ± 0.17		0.95 ± 0.05	1.42 ± 0.14	1.00 ± 0.05	1.00 ± 0.10	5 µM
1 µM	0.79 ± 0.05	0.80 ± 0.05	0.98 ± 0.00	1.19 ± 0.05	***GR2***	1.06 ± 0.10	0.82 ± 0.03	1.09 ± 0.03	0.97 ± 0.08	1 µM
5 µM	0.81 ± 0.03	0.77 ± 0.05	0.83 ± 0.06	0.95 ± 0.10		1.12 ± 0.04	0.64 ± 0.03	0.99 ± 0.05	0.92 ± 0.03	5 µM
	Oxidative stress markers									
1 µM	2.82 ± 1.00	2.38 ± 0.12	1.66 ± 0.18	7.03 ± 1.62	***UPOX***	1.62 ± 0.15	0.89 ± 0.04	1.26 ± 0.16	1.25 ± 0.12	1 µM
5 µM	2.66 ± 0.30	4.86 ± 0.51	2.61 ± 0.08	13.8 ± 1.12		3.46 ± 0.55	1.03 ± 0.04	1.98 ± 0.33	1.38 ± 0.07	5 µM
1 µM	5.34 ± 1.80	9.19 ± 2.44	15.4 ± 7.40	6.65 ± 2.53	***defensin-like***	0.83 ± 0.05	11.6 ± 2.16	1.14 ± 0.21	1.11 ± 0.26	1 µM
5 µM	12.3 ± 4.59	85.3 ± 5.31	12.2 ± 4.39	69.1 ± 6.62		2.94 ± 0.89	21.0 ± 2.98	1.16 ± 0.16	3.48 ± 1.13	5 µM
1 µM	2.43 ± 0.79	10.9 ± 1.06	2.38 ± 0.26	5.52 ± 2.00	***AT1G-19020***	0.38 ± 0.14	3.36 ± 0.41	1.05 ± 0.19	0.84 ± 0.12	1 µM
5 µM	5.00 ± 1.33	16.2 ± 1.23	4.24 ± 0.55	11.6 ± 2.12		0.60 ± 0.05	6.53 ± 0.99	1.16 ± 0.19	1.02 ± 0.23	5 µM
1 µM	1.06 ± 0.04	1.87 ± 0.06	1.41 ± 0.13	2.10 ± 0.22	***AT1G-05340***	0.09 ± 0.01	5.67 ± 0.13	1.60 ± 0.61	0.96 ± 0.14	1 µM
5 µM	1.29 ± 0.08	2.52 ± 0.44	1.77 ± 0.13	1.65 ± 0.23		0.27 ± 0.04	22.0 ± 0.13	1.14 ± 0.14	1.91 ± 0.89	5 µM
1 µM	2.58 ± 1.07	6.01 ± 0.57	1.82 ± 0.14	7.80 ± 3.10	***TIR-class***	0.21 ± 0.14	2.96 ± 0.27	1.08 ± 0.35	0.71 ± 0.12	1 µM
5 µM	6.54 ± 2.00	15.4 ± 1.39	5.57 ± 1.49	33.2 ± 2.81		0.20 ± 0.04	3.83 ± 0.55	0.95 ± 0.14	0.86 ± 0.17	5 µM

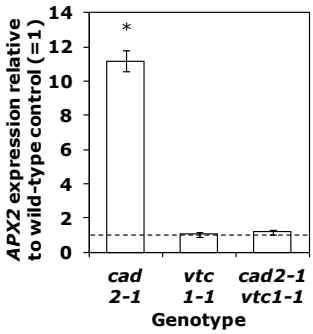


Fig. 5.8 *Gene expression of ascorbate peroxidase2 (APX2) in leaves of three Arabidopsis thaliana mutants (cad2-1, vtc1-1 and cad2-1 vtc1-1) under control conditions. Gene expression is relatively expressed to wild-type control (1: dashed line, n = 4, two-way ANOVA, significance level: * P < 0.05).*

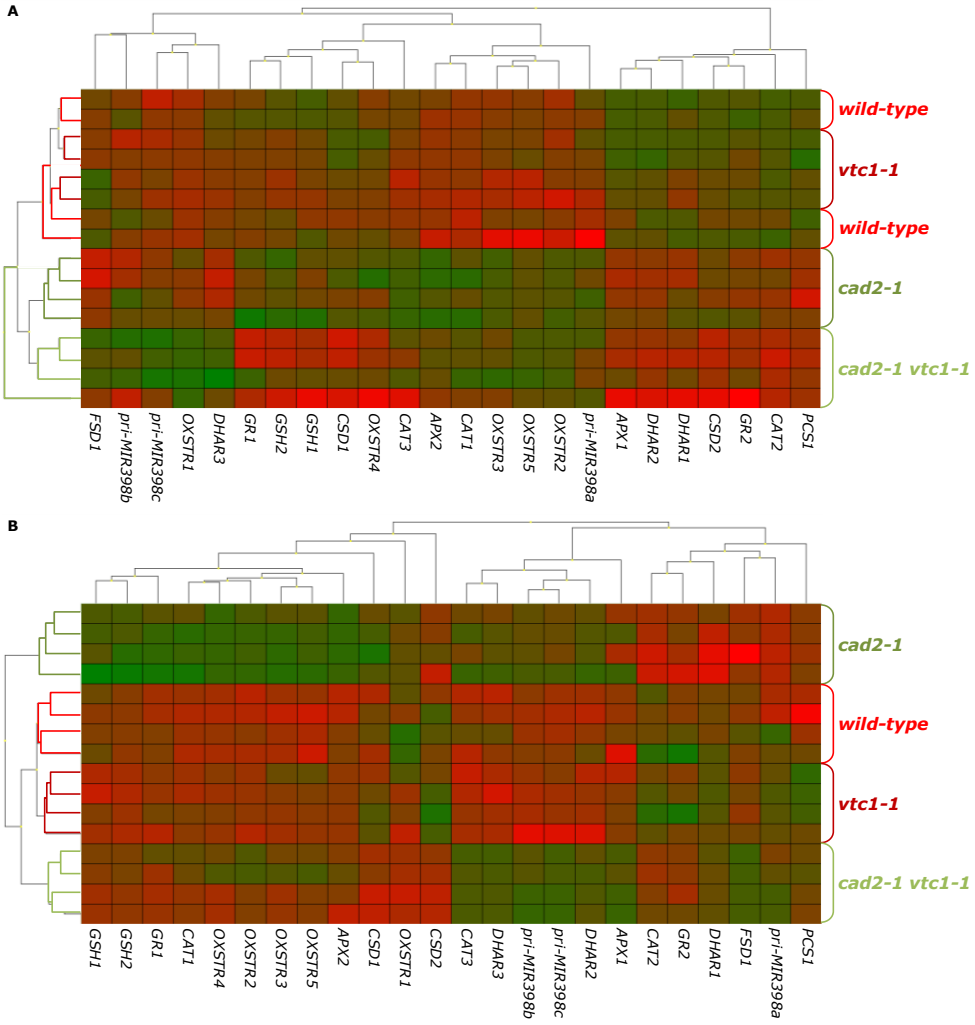


Fig. 5.7 *Heat map representations of the gene expression data collected from roots (A) and leaves (B) of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 5 µM $CdSO_4$ for 72 h in hydroponics (n = 4). Hierarchical clustering of genes is shown on the top and bottom, genotype clustering is visualised on the left and right. Green shaded boxes indicate high and red shaded boxes low gene expression. Genes: γ-glutamylcysteine synthetase (GSH1), glutathione synthetase (GSH2), phytochelatin synthase (PCS1), iron superoxide dismutase (FSD1), copper/zinc superoxide dismutase (CSD1/2), primary microRNA398 transcripts (pri-MIR398a-b/c), catalase (CAT1-3), ascorbate peroxidase (APX1/2), dehydroascorbate reductase (DHAR1-3), glutathione reductase (GR1/2), Toll-Interleukin-1 class (TIR), up-regulated by oxidative stress (UPOX).*

5.5 Discussion

Upon Cd toxicity, plants activate their defence strategies related to both the cellular free metal content and the regulation of antioxidative responses. Glutathione plays a key role as chelator due to the high affinity of Cd for its thiol group and as a precursor for PCs. Antioxidative mechanisms are largely based on the use and recycling of both GSH and AsA, complemented by SOD and CAT. In addition to their function as primary antioxidant, GSH and AsA act as a substrate for the regeneration of other essential antioxidants. Together, these functions influence the cellular redox state of free reduced metabolites, which includes both concentration and reduction/oxidation ratio (Jozefczak *et al.* 2012; Bielen *et al.* 2013). Therefore, both parameters concerning GSH and AsA redox state were taken into consideration in *Arabidopsis thaliana* plants exposed to environmentally realistic Cd concentrations (1 and 5 µM) during 72 h (for a schematic overview, see Fig. 5.9) (Krznaric *et al.* 2009). In general, Cd-exposed roots of wild-type plants were in a more stressed state than leaves. This was apparent by a shift towards a more oxidised cellular redox state (Fig. 5.3 and 5.4) and the induction of several oxidative stress marker genes (Table 5.2) in the roots, while the leaves tended to a more reducing environment and only one marker gene was up-regulated. Although K leakage has been associated with metal-induced membrane damage (Demidchik *et al.* 2010), no K efflux was observed upon Cd exposure (Fig. 5.2D). This confirms that the roots were able to cope with the elevated metal concentrations (Keunen *et al.* 2011b). In agreement with previous findings, they responded via increasing both their chelating capacity via PC production (Fig. 5.5) and their antioxidative capacity via up-regulation of $O_2^{\bullet-}$-scavenging *FSD1* and H_2O_2-scavenging *APX2* genes (DalCorso *et al.* 2008; Jozefczak *et al.* 2014). Cadmium retention and detoxifica-

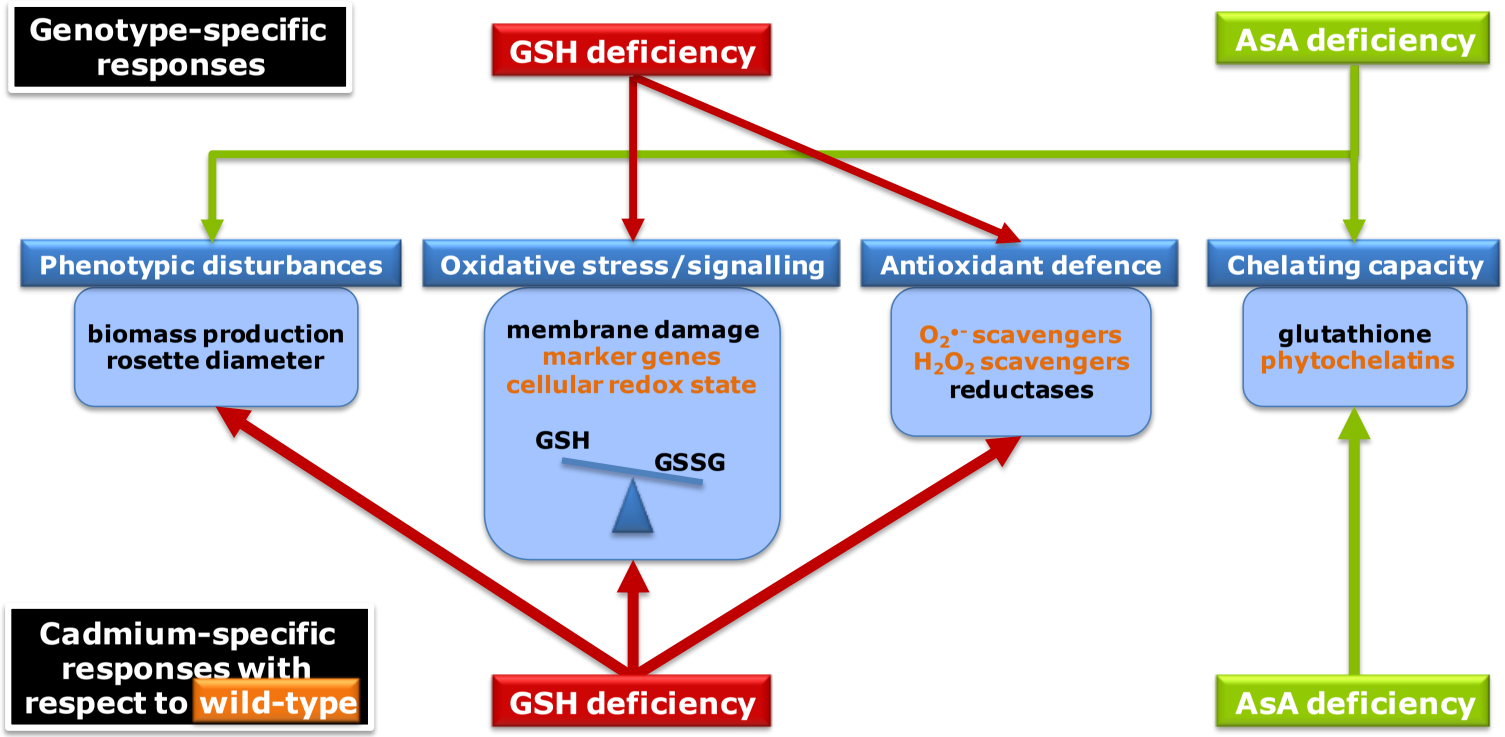


Fig. 5.9 *Schematic overview of both genotype- (top) and cadmium (Cd)-specific (bottom) responses. Wild-type responses under Cd-stress are indicated in orange. Red and green arrows represent additional responses with respect to the wild-type responses in mutants deficient in glutathione (GSH) and ascorbate (AsA), respectively. Abbreviations: glutathione disulfide (GSSG), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2).*

tion in roots contributed to lower Cd levels in the leaves (Fig. 5.2A). Together with high levels and highly reducing redox potentials concerning GSH and AsA, a well-balanced cellular redox state and an optimal function of the AsA-GSH cycle was reflected in the leaves of wild-type plants. Several studies reported increased enzyme activities concerning this cycle, highlighting its relevance in Cd defence (Paradiso *et al.* 2008; Drazkiewicz *et al.* 2010).

5.5.1 Cadmium-sensitive glutathione deficient mutants perceive permanent oxidative stress

Glutathione and AsA are abundant and multifunctional metabolites in plants. Deficiency in any of these two metabolites was investigated by including mutants (*cad2-1*, *vtc1-1* and *cad2-1 vtc1-1*) in this study, which demonstrated multiple genotype-specific effects (for a schematic overview, see Fig. 5.9). Although the GSH deficient *cad2-1* mutant did not exhibit any phenotype, both GSH deficient mutants suffered from oxidative stress under control conditions. On the one hand, the cellular redox state was shifted towards a more oxidised GSH pool in comparison to wild-type plants (Fig. 5.4). It has been widely accepted that GSH is a key molecule of the cellular redox homeostasis through its total concentration as well as its reduced/oxidised ratio. Several studies suggested that changes in the GSH redox state regulate multiple cellular processes at gene expression and protein level, as demonstrated by the elevated *APX2* expression in the leaves of *cad2-1* plants (Cobbett *et al.* 1998; Mou *et al.* 2003; Parisy *et al.* 2007; Jozefczak *et al.* 2012). On the other hand, elevated transcript levels of oxidative stress marker genes were observed: *UPOX* and the *defensin-like* gene (Fig. 5.6) (Gadjev *et al.* 2006). This confirms that the GSH redox state is an important indicator of a cell's biological status, and thus reflects its cellular toxicity (Schäfer & Buettner 2001). Recent *in vivo* studies using a redox-sensitive fluorescent probe in GSH deficient mutants associated increased susceptibility to pathogens with a more oxidised GSH redox potential under normal conditions (Parisy *et al.* 2007; Dubreuil-Maurizi *et al.* 2011). Therefore, we suggest that both decreased GSH levels and a more oxidised GSH pool result in elevated oxidative stress, which contributes to increased sensitivity to Cd.

In both plant organs total GSH levels and the GSH/GSSG ratio were lower in the *cad2-1* mutant than in wild-type plants under control conditions, indicative for a

more oxidised cellular environment. Under Cd exposure, GSH-deficient mutants showed a lower Cd translocation to the shoots (Fig. 5.2B), which is in agreement with a recent study (Sobrino-Plata *et al.* 2014). Nevertheless, this resulted in elevated toxicity responses and oxidative stress upon Cd exposure. Symptoms were defined as severe reductions in root and leaf fresh weight (Table 5.1), increased transcript levels of oxidative stress marker genes (Table 5.2), and an even more oxidised GSH redox state in roots (Fig. 5.4) upon Cd exposure. Similar to wild-type plants, the roots of *cad2-1* mutants were generally more stressed than leaves. The more oxidised environment in the mutant under control conditions, contributed to the activation of alternative pathways using $O_2^{\bullet -}$- as well as H_2O_2-scavengers in order to adapt to the increasing Cd levels, while wild-type plants with a higher level of total GSH and GSH/GSSG ratio did not. These findings support the fact that changes in the cellular redox state act on the regulation of gene expression through oxidation or reduction of transcription factors (Apel & Hirt 2004; Mittler *et al.* 2004). In general, a decreased capacity to chelate Cd ions, in combination with a more oxidised GSH pool, resulted in the activation of antioxidative defence systems upon Cd exposure, which were not required under wild-type conditions. Together, these data confirm the essential role for the GSH redox state in elevated sensitivity to Cd exposure in GSH deficient mutants (Howden *et al.* 1995a; Jozefczak *et al.* 2012).

5.5.2 Elevated thiol levels in ascorbate deficient plants protect them against cadmium stress

The importance of AsA and its precursors in cell proliferation, elongation and cell wall synthesis (Veljovic-Jovanovic *et al.* 2001; Bielen *et al.* 2013), was confirmed by the slow-growth phenotypes of the AsA deficient mutants (Fig. 5.1, Table 5.1). Despite decreased AsA levels, *vtc1-1* plants showed a reduced cellular redox state similar to wild-type plants under control conditions (Fig. 5.3 and 5.4), which was confirmed by the lack of oxidative stress marker gene inductions (Fig. 5.6). An interesting difference with wild-type plants however, was the elevated thiol concentration detected in unexposed *vtc1-1* plants (Fig. 5.5). Increased basal GSH levels have been observed before in these mutants (Veljovic-Jovanovic *et al.* 2001; Pavet *et al.* 2005). In addition, AsA deficiency was previously suggested to provide a primed state that decreases pathogen

susceptibility in *A. thaliana*. This priming was related to elevated levels of phytohormones, *i.e.* components involved in developmental and defence signalling (Pastori *et al.* 2003; Barth *et al.* 2004; Pavet *et al.* 2005; Mukherjee *et al.* 2010; Brosché & Kangasjarvi 2012). In this study, we hypothesise that AsA deficiency also prepares *A. thaliana* plants for abiotic stresses like Cd exposure by stimulating their thiol production.

In general, *vtc1-1* mutants showed a similar transcript profile as wild-type plants concerning both the antioxidant and oxidative stress marker genes (Table 5.2). Additionally, these mutants were more able than wild-type plants to keep a stable and reducing environment concerning AsA and GSH redox state (Fig. 5.3, 5.4), indicative of an active recycling of both metabolites (Conklin & Barth 2004). Together, these data support the hypothesis about AsA deficiency rendering *Arabidopsis* mutants less sensitive to Cd. In addition to the elevated thiol levels found under control conditions, PC levels were approximately twice as high in the roots of *vtc1-1* than in wild-type plants upon Cd exposure (Fig. 5.5). As one of the major defence mechanisms in Cd-exposed plants is fast chelation and sequestration by thiols (Zhang *et al.* 2013; Jozefczak *et al.* 2014), the enhanced chelating capacity in *vtc1-1* plants is suggested to contribute to a less Cd-sensitive genotype.

5.5.3 Conclusion

A recent publication summarised that low AsA conditions led to opposite phenotypes to low GSH conditions after multiple stresses including ozone treatment and pathogen infection (Brosché & Kangasjarvi 2012). Our observations indicate that both GSH and AsA deficiency alter plant GSH homeostasis in the absence of Cd stress, resulting in opposite Cd sensitivities relative to wild-type plants. Glutathione deficiency on the one hand, resulted in a more oxidised GSH redox state and elevated oxidative stress under control conditions. Upon Cd exposure, GSH-deficient mutants were hampered in chelation, suffered more oxidative stress and therefore activated multiple alternative pathways such as SOD, CAT and APx, indicating a higher Cd sensitivity. Ascorbate deficiency on the other hand, was associated with elevated basal thiol levels. Enhanced PC synthesis upon Cd exposure, in comparison to wild-type plants, contributed to decreased sensitivity towards Cd in AsA deficient mutants (for a schematic overview, see Fig. 5.9).

Appendices

Table A.5.1 *List of primers used in the Reverse Transcription quantitative PCR (RTqPCR). Exon-Exon-junction (E-E-jn), Intron-Exon-junction (In-E-jn).*

Gene	TAIR: locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
APX1	*AT1G07890*	Ascorbate peroxidase1	FW: TGCCACAAGGATAGGTCTGG REV: CCTTCCTTCTCTCCGCTCAA	Exon 5	101 bp	96.3 %
APX2	*AT3G09640*	Ascorbate peroxidase2	FW: TTGCTGTTGAGATCACTGGAGGA REV: TGAGGCAGACGACCTTCAGG	Exon 3	91 bp	90.9 %
CAT1	*AT1G20630*	Catalase1	FW: AAGTGCTTCATCGGGAAGGA REV: CTTCAACAAAACGCTTCACGA	E5-E6-jn	103 bp	97.6 %
CAT2	*AT4G35090*	Catalase2	FW: AACTCCTCCATGACCGTTGGA REV: TCCGTTCCCTGTCGAAATTG	Exon 2	76 bp	98.3 %
CAT3	*AT1G20620*	Catalase3	FW: TCTCCAACAACATCTCTTCCCTCA REV: GTGAAATTAGCAACCTTCTCGATCA	Exon 2	91 bp	95.6 %
CSD1	*AT1G08830*	CuZn superoxide dismutase1	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5	102 bp	93.8 %
CSD2	*AT2G28190*	CuZn superoxide dismutase2	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6	101 bp	93.9 %
DHAR1	*AT5G16710*	Dehydroascorbate reductase1	FW: CCAGATTCACTTCCTTTCGTCAA REV: TTACATCCTCTGTTTCCGCCC	Exon 6	91 bp	94.0 %
DHAR2	*AT1G75270*	Dehydroascorbate reductase2	FW: ATCAGATGGGTCTTGTAGGGAAGC REV: GTGCTCCTGATGTTCTCGGC	Exon 2	91 bp	95.1 %
DHAR3	*AT1G19550*	Dehydroascorbate reductase3	FW: AACTCTTTCCCCGGCGATAA REV: CTGAATTTGCCTCTGTTGGCTC	Exon 1	92 bp	95.2 %
FSD1	*AT4G25100*	Fe superoxide dismutase1	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4	101 bp	88.8 %
GR1	*AT3G24170*	Glutathione reductase1	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15	101 bp	94.8%
GR2	*AT3G54660*	Glutathione reductase2	FW: GCCCAGATGGATGGAACAGAT REV: TAGGGTTGGAGAATGTTGGCG	Exon 5	91 bp	96.4 %
GSH1	*AT4G23100*	γ-Glutamylcysteine synthetase	FW: CCCTGGTGAACTGCCTTCA REV: CATCAGCACCTCTCATCTCCA	Exon 5	101 bp	98.6 %
GSH2	*AT5G27380*	Glutathione synthetase	FW: GGACTCGTCGTTGGTGACAA REV: TCTGGGAATGCAGTTGGTAGC	Exon 11	101 bp	92.6 %

Table A.5.1 *Continued.*

Gene	TAIR: locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
pri-MIR398a	*AT2G03445*	Primary microRNA398a	FW: AGAAGAAGAGAAGAACAACAGGAGGTG REV: ATTAGTAAGGTGAAAAAATGG	In1-E1-jn	156 bp	96.5 %
pri-MIR398b	*AT5G14545*	Primary microRNA398b	FW: AGTAATCAACGGCTGTAATGACGCTAC REV: TGACCTGAGAACACATGAAAACGAGAG	Exon 1	67 bp	85.3 %
pri-MIR398c	*AT5G14545*	Primary microRNA398c	FW: TCGAAACTCAAACTGTAACAGTCC REV: ATTTGGTAAATGAATAGAAGCCACGGGCCACG	Exon 1	241 bp	104.2 %
OXSTR1	*AT2G21640*	Oxidative stress marker1	FW: GACTTGTTTCAAAAACACCATGGAC REV: CACTTCCTTAGCCTCAATTTGCTTC	Exon 1	91 bp	95.9 %
OXSTR2	*AT2G43510*	Oxidative stress marker2	FW: ATGGCAAAGGCTATCGTTTCC REV: CGTTACCTTGCGCTTCTATCTCC	Exon 1	91 bp	96.8 %
OXSTR3	*AT1G19020*	Oxidative stress marker3	FW: GAAAATGGGACAAGGGTTAGACAAA REV: CCCAACGAAAACCAATAGCAGA	Exon 1	92 bp	95.7 %
OXSTR4	*AT1G05340*	Oxidative stress marker4	FW: TCGGTAGCTCAGGGTAAAGTGG REV: CCAGGGCACAACAGCAACA	Exon 2	91 bp	99.1 %
OXSTR5	*AT1G57630*	Oxidative stress marker5	FW: ACTCAAACAGGCGATCAAAGGA REV: CACCAATTCGTCAAGACAACACC	Exon 1	91 bp	97.3 %
PCS1	*AT5G44070*	Phytochelatin synthase1	FW: TGCCAAGGAGCTGAAATCTT REV: ACCGTGCCTTCAGAGTCATC	Exon 2	91 bp	95.1 %
PPR	*AT5G55840*	Pentatricopeptide repeat protein	FW: AAGACAGTGAAGGTGCAACCTTACT REV: AGTTTTTGAGTTGTATTTGTCAGAGAAAG	Intron 2	59 bp	81.5 %
SAND	*AT2G28390*	SAND family	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13	61 bp	107.8 %
Tip41	*AT4G34270*	Tip41-like protein	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn	61 bp	91.2 %
UBC	*AT5G25760*	Ubiquitin conjugating enzyme	FW: CTGCGACTCAGGGAATCTTCTAA REV: TTGTGCCATTGAATTGAACCC	E3-E4-jn	61 bp	98.7 %

Table A.5.2 *Reverse Transcription quantitative PCR (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines derived from Bustin et al. 2009.*

Sample/Template	
Source	*Arabidopsis thaliana* roots or shoots in a hydroponic culture
Method of preservation	harvest in liquid N_2 then stored at -80°C
Storage time	three weeks
Handling	frozen
Extraction method	columns: RNAqueous Total RNA Isolation Kit* (Ambion, Lennik, Belgium)
RNA: DNA-free	TURBO DNA-free Kit* (Ambion, Lennik, Belgium) intron-spanning primers and verification of single peak on melt curves
Concentration	NanoDrop®: ND-1000 Spectrofotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Microfluidics: Bioanalyzer* (Agilent Technologies, Waldbronn, Duitsland) for a representative subset of the samples
Assay optimisation/validation	
Accession number	see Table A.5.1
Amplicon details	exon location & amplicon size see Table A.5.1
Primer sequence	see Table A.5.1
In silico	Primer-BLAST
Empirical	primer concentration (300 nM) annealing temperature (60°C)
Priming conditions	random hexamer priming
PCR efficiency	dilution curves (slope, y-intercept & r^2, see Table A.5.1)
Linear dynamic range	samples are within the efficiency curve
RT/PCR	
Protocols	TURBO DNA-free Kit* (Ambion) Fast SYBR Green* (Applied Biosystems, Paisley, UK) see Materials and Methods
Reagents	see Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software version 1.4 (Applied Biosystems, Lennik, Belgium, 2001-2006)
Statistical justification	4 biological replicates, two-way ANOVA and Tukey post-hoc adjustment for multiple comparison
Normalisation	3 reference genes selected using geNorm, version 3.5 (Centre for Medical Genetics, Ghent, Belgium, 2001-7)

*All procedures were performed according to the manufacturer's protocol.

Chapter 6

Thiols play a regulatory role *in copper/zinc superoxide dismutase* expression during cadmium exposure in *Arabidopsis thaliana*

Research article – in preparation for Plant Physiology

6.1 Abstract

Glutathione (GSH) and superoxide dismutase (SOD) are involved in the fast responses upon cadmium (Cd) exposure in *Arabidopsis thaliana*. Glutathione is essential for its Cd chelating as well as antioxidant properties via the thiol group on cysteine. Early SOD activation reduces the primary reactive oxygen species (ROS) formed. Three main families of SODs are distinguished in plants, according to the metal in the active centre: CuZnSOD, FeSOD and MnSOD. Exposure to Cd resulted in transcriptional SOD responses in wild-type plants that are associated with the activation of the transcription factor squamosa promoter-binding protein-like 7 (SPL7). This transcription factor stimulates the expression of both *FSD1* and microRNA398 (*MIR398*), the latter resulted in *CSD1* and *CSD2* repression. Three GSH deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) showed a similar SPL7-regulated response upon Cd exposure. However, the mutants revealed a fast but transient Cd-induced transcriptional activation of *CSD1/2* transcripts, overruling their repression by MIR398. This study suggests that the (subcellular) thiol redox state contributes to the transcription rate of *CSD* genes upon Cd exposure, pointing towards an unknown factor regulating *CSD* expression.

6.2 Introduction

Glutathione (GSH) is an essential metabolite in plant metabolism and abiotic stress responses like exposure to cadmium (Cd). It is the predominant non-protein thiol in plant cells with multiple functions. On the one hand, GSH is involved in redox signalling and the antioxidative defence (Noctor & Foyer 1998b; Foyer & Noctor 2011). On the other hand, GSH represents the precursor for phytochelatins (PCs) (Cobbett & Goldsbrough2002) and a major storage and transport form of reduced sulfur in plants (Kopriva & Rennenberg 2004). Glutathione biosynthesis is conserved in all organisms and involves only two enzymes: γ-glutamylcysteine synthetase (GSH1), which forms a peptide bond between γ-glutamate and cysteine; and glutathione synthetase (GSH2), which catalyses the addition of a glycine residue. Both enzymes are encoded by single genes in *Arabidopsis* and the rate-limiting step in GSH biosynthesis is catalysed by GSH1 (Foyer & Noctor 2005a; Jozefczak *et al.* 2012; Noctor *et al.* 2012). The

activity of GSH1 is tightly regulated at three levels: GSH itself exerts a negative feedback (Hell & Bergmann 1990); a repressor that binds to the *GSH1* promoter, which is released upon oxidation (Xiang & Bertrand 2000); and post-translational redox controls (May *et al.* 1998). It has been indicated that expression of both synthesis genes is induced in response to Cd stress (Xiang & Oliver 1998; Jozefczak *et al.* 2014). Since mutational knockouts in one of both genes result in lethal phenotypes (Cairns *et al.* 2006) and GSH1 is the rate-limiting-step, GSH deficient conditions were investigated in mutants with decreased GSH1 activity. Known mutations in the *Arabidopsis thaliana* GSH1 gene affect residues in regions critical for substrate binding and catalysis, explaining GSH deficiency in these mutants. The cadmium-sensitive mutant (*cad2-1*) has been mapped to a 6-bp deletion in the GSH1 gene, which is suggested to alter the position of residues involved in substrate binding. Depressed GSH levels in the *A. thaliana* phytoalexin-deficient 2-1 mutant (*pad2-1*) are caused by a substitution close to the active centre. Finally, the regulator of APX2 1-1 mutant (*rax1-1*) suffers from impaired cysteine binding due to a missense mutation (Hothorn *et al.* 2006; Parisy *et al.* 2007).

Together with GSH, superoxide dismutase (SOD) is involved in a fast response upon Cd-induced oxidative stress (Chapter 4; Jozefczak *et al.* 2014). This enzyme is responsible for the reduction of superoxide ($O_2^{\bullet -}$), the primary reactive oxygen species (ROS) formed in the univalent reduction of O_2. Superoxide dismutase converts $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2), initiating the production of other ROS (Suzuki *et al.* 2012). Depending on the metal present in the active site, three SOD families are distinguished in plants: CuZnSOD, FeSOD and MnSOD. In the genome of *Arabidopsis*, seven SOD genes have been identified including three isoforms of both FeSOD (plastidal *FSD1-3*) and CuZnSOD (cytosolic *CSD1*, plastidal *CSD2* and peroxisomal *CSD3*) and a single isoform of MnSOD (mitochondrial *MSD1*) (Kliebenstein *et al.* 1998). One FeSOD (*FSD1)* and two CuZnSOD (*CSD1/2*) genes are known to be regulated by the squamosa promoter-binding protein-like 7 (SPL7). Activation of this transcription factor results in up-regulation of *FSD1* and microRNA398 (*MIR398*). The latter targets and hence represses both *CSD1* and *CSD2* transcripts (Yamasaki *et al.* 2007; Nagae *et al.* 2008). Recent studies demonstrated activation of this response in Cd-exposed wild-type plants at transcriptional level (Cuypers *et al.*

2011), whereas an opposite regulation of *CSD* isoforms was observed in *cad2-1* mutants after exposure to Cd for 24 h (Chapter 4). Therefore, this study focuses on the Cd-induced *CSD* regulation in *A. thaliana* plants under GSH deficient conditions. In this regard, multiple GSH deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) were exposed to Cd during 24 and 72 h. This regulation was investigated at transcriptional, posttranscriptional and enzyme level in order to shed more light on the underlying mechanisms.

6.3 Materials and methods

6.3.1 Experimental design and methodology

Arabidopsis thaliana (Col-0 ecotype) seedlings of wild-type (Lehle seeds, Round Rock TX, USA) plants were cultivated in parallel with three GSH-deficient genotypes mutated in *GSH1* (*AT4G23100*). The Cd-sensitive *cad2-1* mutant (carrying a deletion of codons 237 and 238, and a mutation at codon 239 causing a valine to leucine change) was a gift of Dr. Christopher Cobbett (Melbourne University, Australia; Cobbett *et al.* 1998). The phytoalexin-deficient *pad2-1* mutant (carrying a serine to asparagine change at codon 298; Parisy *et al.* 2007) was obtained from the European *Arabidopsis* Stock Centre (uNASC). The regulator of *ASCORBATE PEROXIDASE 2* mutant *rax1-1* (carrying an arginine to lysine change at codon 228; Ball *et al.* 2004) was kindly donated by Prof. Dr. Phil Mullineaux (University of Essex, England).

Seeds were surface sterilised and grown in hydroponic culture in a climate chamber with the following conditions: 12 h photoperiod; 22/18°C day/night regime; 65% relative humidity; and 170 µmol m^{-2} s^{-1} light at the rosette level, provided by a combination of blue, red and far-red Philips Green-Power LED modules, simulating the photosynthetically active radiation (PAR) of sunlight (Chapter 3). The nutrient solution was refreshed every three or four days. After three weeks, the hydroponic solution was supplied with 0 or 5 µM $CdSO_4$. After 24 and 72 h, the leaves and roots were separated and dried prior to element determination or snap frozen in liquid nitrogen, then stored at -80°C.

6.3.2 Element determination

At the time of harvest, roots were washed with ice-cold 10 mM $Pb(NO_3)_2$ (15 min, 4°C) and thoroughly rinsed with distilled water. Leaves were only washed in distilled water. The fresh material was dried at 60°C during three weeks, and weighed before digestion in 70-71% HNO_3 with a heat block. Element levels were measured via inductively coupled plasma-optical emission spectrometry (ICP-OES 710, Agilent Technologies, Australia).

6.3.3 Metabolite determination

Non-protein thiols were extracted and analysed after derivatisation with monobromobimane (mBrB) by reverse-phase HPLC according to Huguet *et al.* (2012). In brief, 25 mg aliquots of frozen material were ground in 0.1% trifluoroacetic acid, 6.3 mM DTPA (pH ≤ 1) and 10 mM Nacetyl-l-cysteine (internal standard, NAC) and subsequently filtered and incubated with 280 mM mBrB, 125 mM HEPPS (pH 8.2) and 4 mM DTPA (30 min, 45°C, dark). Thiols were separated on a Nova-Pak C_{18} analytical column (Waters, Milford, USA) at 37° and eluted with a slightly concave gradient of water and methanol. Fluorescent compounds were identified with Ellman's reagent using a Waters 474 fluorescence detector. Quantification was based on NAC and GSH standards (0-20 mM) and corrected for derivatisation efficiency (Huguet *et al.* 2012).

Total GSH and AsA were extracted and analysed by enzymatic assays on a plate reader based on the protocol previously described by Queval and Noctor (2007b). In brief, frozen samples (50 mg root, 75 mg leaf) were ground in 200 mM HCl and adjusted to pH 4.5. Ascorbate was incubated with 1 mM DTT (15 min, 20°C) and adjusted to pH 5.6. Next, AsA levels were determined spectrophotometrically at 256 nm after addition of 1 U ml^{-1} ascorbate oxidase (from Curcubita sp., Sigma, St. Louis, MO, USA) and corrected for background signal. Total GSH was measured using a kinetic enzymatic recycling assay based on the GSH-dependent reduction of Ellman's reagent (600 mM) and subsequent reduction of glutathione disulfide (GSSG) by GR (1 U ml^{-1}) in the presence of NADPH (500 mM). The rate of absorbance change over 5 min is proportional to the GSH concentration which was calculated using a GSH standard curve (0-50 µM) (Queval & Noctor 2007b).

6.3.4 Enzyme activity measurements

Frozen root (150 mg) or leaf (200 mg) tissues were ground in liquid nitrogen and agitated (30 min, 4°C) in 2 ml ice-cold 0.1M Tris-HCl buffer (pH 7.8) containing 5 mM EDTA, 5 mM dithioerythritol, 1% polyvinylpyrrolidone and 1% Nonidet P40. After centrifugation (48 384 *g*, 30 min, 4°C), the proteins in the supernatant were first precipitated in 40% and subsequently in 80% ammonium sulphate. The pellet was resuspended in 1.75 ml 25 mM Tris-HCl (pH 7.8) and purified in PD-10 desalting columns (GE Healtcare, UK) by centrifugation (1 578 *g*, 2 min, 4°C). The protein extracts were stored at -70°C for further analysis. Due to APx instability, another extraction method was used to measure APx activity: frozen root (100 mg) and leaf (200 mg) tissues were homogenised with 5 mg insoluble polyvinylpyrrolidone in 2 ml ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM DDT, 1 mM EDTA and 10 mM Na-AsA and centrifuged (10 min, 16 100 *g*, 4°C). These samples were not stored but immediately used for analysis.

The enzyme activities were measured spectrophotometrically in the supernatant at 25°C. Glutathione reductase (GR, EC 1.6.4.2) activity measurement was based on the reduction of GSSG in the presence of NADPH at 340 nm and catalase (CAT, EC 1.11.1.6) activity was measured at 240 nm (Bergmeyer *et al.* 1974). Activity measurement of APx (EC 1.11.1.11) was based on AsA oxidation, which was measured at 298 nm (Gerbling *et al.* 1984). Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was measured at 340 nm (Bergmeyer *et al.* 1974). Determination of SOD (EC 1.15.1.1) activity was based on the inhibition of cytochrome *c* at 550 nm (McCord & Fridovich 1969). Peroxidase (POD, EC 1.11.1.9) activity was determined using guaiacol or syringaldazine as a substrate. Guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD) activities were measured at 436 and 530 nm, respectively (Bergmeyer *et al.* 1974; Imberty *et al.* 1984).

6.3.5 Gene expression analysis

Samples were disrupted under frozen conditions using two stainless steel beads and the Retch Mixer Mill MM 2000 (Retsch, Germany). The RNAqueous Total RNA Isolation Kit (Ambion, Belgium) was used for RNA isolation. Subsequently, the TURBO DNA-free kit (Ambion) and the High Capacity cDNA Reverse

Transcription Kit (Ambion, random hexamer primers and 1 µg RNA input) were used to remove genomic DNA and to carry out reverse transcription, respectively. Tenfold diluted cDNA in 1/10 diluted TE-buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) was stored at -20°C. Quantitative PCR (qPCR) was performed using Fast SYBR Green chemistry according to the manufacturer's instructions on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Belgium). Relative gene expression was calculated as $2^{-\Delta Cq}$ and normalised with a normalisation factor based on the expression of the following reference genes: *AT5G55840*, *AT4G34270* and *AT2G28390* for roots and *AT5G25760*, *AT4G34270* and *AT2G28390* for leaves (Remans *et al.* 2008). Gene-specific primers (300 nM, Table A.6.1 in Appendices) were designed and optimised using Primer Express (Applied Biosystems). The Reverse Transcription quantitative (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines were taken into account (Table A.6.2 in Appendices) (Bustin *et al.* 2009).

The relative abundance of distinct gene family members was calculated by determining the expression level of each family member in the control sample (wild-type 24 h, 0 µM Cd) relative to the lowest expressed family member. This results in a relative abundance factor for each member of the family, which is used to calculate its relative abundance in the different conditions. Next, the changes in expression level for each member were determined in function of the genotype and Cd concentration used and set relatively to the control (wild-type 24 h, 0 µM Cd).

6.3.6 Statistical analysis

All data were analysed with general linear two-way ANOVA models (Verbeke & Molenberghs 2000). Normal distribution and homoscedasticity were tested using the Shapiro-Wilk and Bartlett test, respectively. Tukey post-hoc adjustment was used to correct for multiple comparison. Logarithmic transformations were applied when necessary to approximate normality. The datasets in which each condition was normalised against its representative control were analysed with one-way ANOVA. All statistical analyses were performed using R (the R foundation of statistical computing, version 2.15.1). Data are mean values ± standard error (SE) and significance was set at the 5% level ($P < 0.05$).

6.4 Results

Three-week-old *A. thaliana* wild-type and GSH deficient plants (*cad2-1*, *pad2-1* and *rax1-1*) were exposed during 24 and 72 h to a sublethal Cd concentration of 5 µM (Keunen *et al.* 2011b), consistent with Cd concentrations found in pore waters of polluted soil (Krznaric *et al.* 2009). Although both organs showed increased Cd levels, Cd preferentially accumulated in roots in GSH deficient mutants (Fig. 6.1A). The latter was clearly indicated by the significantly lower translocation factor for Cd in all mutants in comparison with wild-type plants (Fig. 6.1B). The increase in Cd content was associated with increasing levels of Cu in roots after 72 h, which was more pronounced in the GSH deficient mutants than wild-type plants (Fig. 6.2A). Potassium (K) levels on the contrary, were decreased after 72 h of Cd exposure, independent on the genotype (Fig. 6.2B). No significant changes were detected in the amounts of Cu and K in the leaves or in other elements in both organs (results not shown). Under control conditions, fresh weight was equal for all genotypes, except *rax1-1* plants showed a significantly decreased root fresh weight in comparison to wild-type plants (Table 6.1). After 24 h of Cd exposure, only root fresh weight of *cad2-1* plants was significantly decreased. Exposure during 72 h however, resulted in significant root and leaf fresh weight reduction in all mutants, except the leaf fresh weight of *rax1-1* plants was not significantly affected.

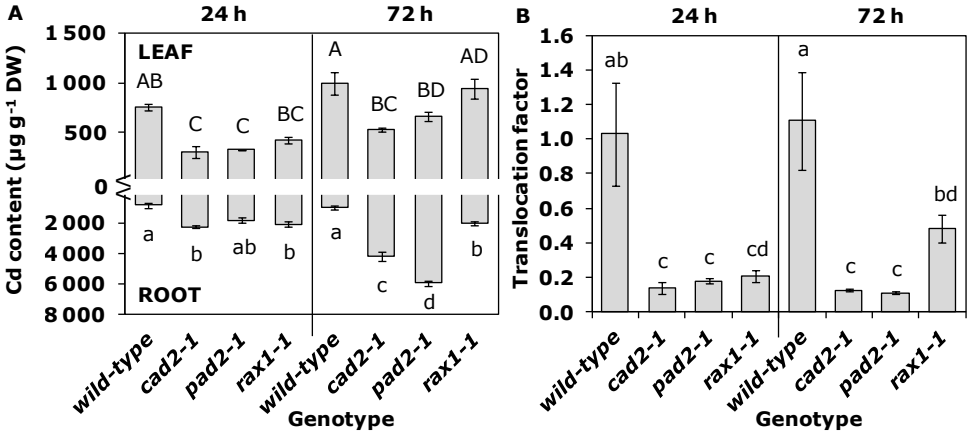


Fig. 6.1 *(A) Root and leaf Cd contents (µg g^{-1} DW) and (B) the corresponding translocation factor from roots to shoots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 5 µM $CdSO_4$ for 24 and 72 h. Statistical significance is expressed using lower (roots and translocation factor) and upper (leaves) case letters; n = 4 (two-way ANOVA, P < 0.05).*

Table 6.1 *Fresh weight (mg FW plant^{-1}) of roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h. Statistical significance is expressed using lower (roots) and upper (leaves) case letters; n = 10 (two-way ANOVA within each time point, P < 0.05).*

	24 h			72 h	
	0 µM Cd	**5 µM Cd**	**genotype**	**0 µM Cd**	**5 µM Cd**
LEAF (mg FW plant^{-1})	65.7 ± 3.3 ^{A}	67.2 ± 3.9 ^{A}	***wild-type***	94.3 ± 7.1 ^{AB}	77.2 ± 6.1 ^{AC}
	72.1 ± 4.2 ^{A}	64.6 ± 5.7 ^{A}	***cad2-1***	## ± 4.4 ^{B}	67.8 ± 5.5 ^{C}
	72.2 ± 4.0 ^{A}	69.1 ± 7.9 ^{A}	***pad2-1***	98.9 ± 7.5 ^{AB}	31.4 ± 1.8 ^{D}
	63.0 ± 7.6 ^{A}	61.1 ± 6.1 ^{A}	***rax1-1***	79.3 ± 4.3 ^{ABC}	74.3 ± 6.0 ^{AC}
ROOT (mg FW plant^{-1})	27.9 ± 3.1 ^{a}	24.7 ± 1.6 ^{ac}	***wild-type***	46.1 ± 4.1 ^{a}	30.9 ± 3.1 ^{ab}
	23.3 ± 1.4 ^{ac}	15.9 ± 1.4 ^{b}	***cad2-1***	43.9 ± 3.8 ^{a}	14.2 ± 1.8 ^{c}
	26.0 ± 2.3 ^{ac}	20.2 ± 1.6 ^{ab}	***pad2-1***	38.5 ± 3.5 ^{ab}	11.7 ± 1.0 ^{c}
	17.3 ± 1.9 ^{bc}	12.9 ± 1.6 ^{b}	***rax1-1***	24.9 ± 2.4 ^{b}	12.2 ± 1.2 ^{c}

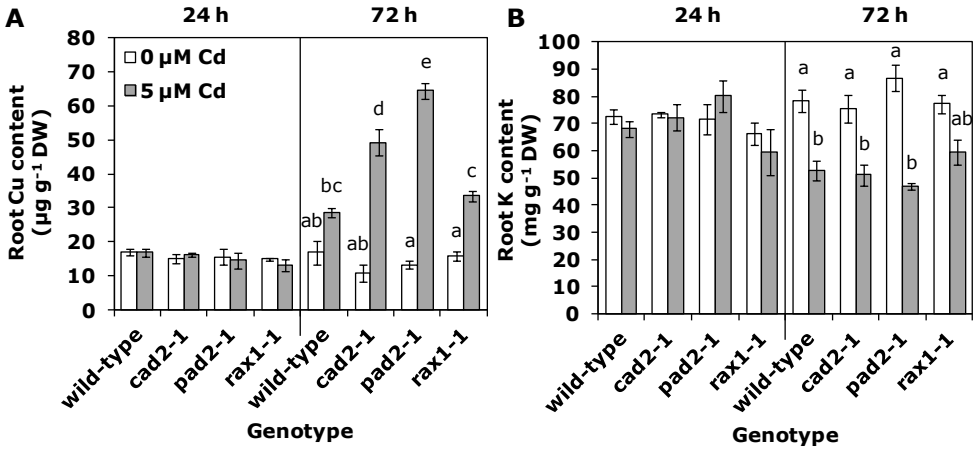


Fig. 6.2 *Root Cu (A) and K (B) contents of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 (□) or 5 (■) µM $CdSO_4$ for 24 and 72 h. Statistical significance is expressed using lower case letters; n = 4 (two-way ANOVA within each time point, P < 0.05).*

6.4.1 Cadmium chelation by phytochelatins is disturbed in glutathione deficient mutants

Mutant genotypes were confirmed by significant deficiencies in GSH levels under control conditions: *cad2-1* plants contained between 20 and 30% of wild-type GSH levels, *pad2-1* mutants showed 15 to 30% and *rax1-1* plants possessed 30 to 40% GSH (Table 6.2A). No significant changes in total GSH content were observed in roots of all Cd-exposed genotypes, although a nearly 50% decrease was noticed in wild-type plants. The roots of all mutants demonstrated an initial

lower GSH/GSSG ratio than wild-type plants under control conditions (Table 6.2B). After 24 h of Cd exposure, the ratio decreased in the roots of all genotypes, but only significantly in wild-type plants. After 72 h in roots, this decrease was no longer significant in wild-type plants, whereas *pad2-1* and *rax1-1* mutants showed an increase in GSH/GSSG levels, which was only significant in *rax1-1* mutants. In leaves, a significant increase in total GSH content could only be observed in *cad2-1* and *pad2-1* mutants after 72 h of Cd exposure. Although no significant changes were detected in the GSH/GSSG ratio in the leaves, an increasing trend was apparent after 72 h of Cd exposure in all genotypes.

The PC profile demonstrated about five- and three-fold increased thiol levels in the roots and leaves of wild-type plants, respectively (Fig. 6.3). Although the wild-type plants produced PCs up to PC5, the mutant plants were also able to produce PCs up to PC4, but to a significantly lesser extent than wild-type plants.

Table 6.2 *(A) Concentrations of total glutathione (GSH) and (B) ratios of GSH/glutathione disulfide (GSSG) in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h. Data are expressed relative to the control condition of wild-type plants (=100.0%). Statistical significance is expressed using lower (roots) and upper (leaves) case letters; n = 6 (two-way ANOVA within each time point, P < 0.05).*

A

TOTAL GSH (µmol g^{-1} FW)	24 h			72 h	
	0 µM Cd	5 µM Cd	genotype	0 µM Cd	5 µM Cd
LEAF	191.8 ± 16.9 ^{A}	203.1 ± 25.6 ^{A}	***wild-type***	246.6 ± 4.3 ^{A}	336.6 ± 45.5 ^{A}
	43.6 ± 6.4 ^{B}	54.6 ± 8.7 ^{B}	***cad2-1***	62.4 ± 9.2 ^{B}	115.3 ± 8.1 ^{C}
	51.9 ± 13.5 ^{B}	37.8 ± 11.5 ^{B}	***pad2-1***	42.7 ± 4.4 ^{D}	138.0 ± 16.7 ^{C}
	75.3 ± 25.9 ^{B}	46.0 ± 3.7 ^{B}	***rax1-1***	76.3 ± 5.2 ^{BE}	106.4 ± 3.2 ^{CE}
ROOT	165.9 ± 24.4 ^{a}	92.3 ± 6.4 ^{a}	***wild-type***	123.7 ± 3.1 ^{a}	66.6 ± 3.5 ^{ab}
	39.8 ± 2.0 ^{bc}	27.5 ± 4.6 ^{b}	***cad2-1***	36.6 ± 6.2 ^{c}	33.1 ± 4.1 ^{c}
	30.7 ± 4.2 ^{bc}	23.1 ± 3.2 ^{b}	***pad2-1***	28.7 ± 4.0 ^{c}	39.6 ± 8.7 ^{bc}
	50.9 ± 6.5 ^{c}	37.3 ± 4.6 ^{bc}	***rax1-1***	35.3 ± 6.4 ^{c}	45.1 ± 7.0 ^{bc}

B

GSH/GSSG	24 h			72 h	
	0 µM Cd	5 µM Cd	genotype	0 µM Cd	5 µM Cd
LEAF	18.5 ± 3.0 ^{A}	34.0 ± 12.6 ^{A}	***wild-type***	4.80 ± 0.62 ^{AB}	21.5 ± 3.9 ^{A}
	15.7 ± 3.1 ^{A}	57.6 ± 18.2 ^{A}	***cad2-1***	6.16 ± 1.44 ^{AB}	20.1 ± 3.5 ^{A}
	25.9 ± 11.0 ^{A}	29.5 ± 8.0 ^{A}	***pad2-1***	4.68 ± 0.63 ^{B}	10.9 ± 4.6 ^{AB}
	27.0 ± 6.0 ^{A}	21.5 ± 4.5 ^{A}	***rax1-1***	5.53 ± 1.37 ^{AB}	13.2 ± 4.4 ^{AB}
ROOT	30.30 ± 3.42 ^{a}	9.55 ± 0.86 ^{b}	***wild-type***	133.9 ± 67.8 ^{a}	46.1 ± 35.0 ^{ab}
	7.20 ± 2.29 ^{bc}	3.65 ± 0.30 ^{bc}	***cad2-1***	31.4 ± 8.1 ^{ab}	6.2 ± 1.2 ^{b}
	3.31 ± 0.63 ^{cd}	2.10 ± 0.19 ^{d}	***pad2-1***	4.5 ± 1.2 ^{bc}	12.3 ± 7.2 ^{bc}
	11.26 ± 3.79 ^{b}	7.34 ± 0.76 ^{bc}	***rax1-1***	2.5 ± 1.2 ^{c}	15.1 ± 4.2 ^{ab}

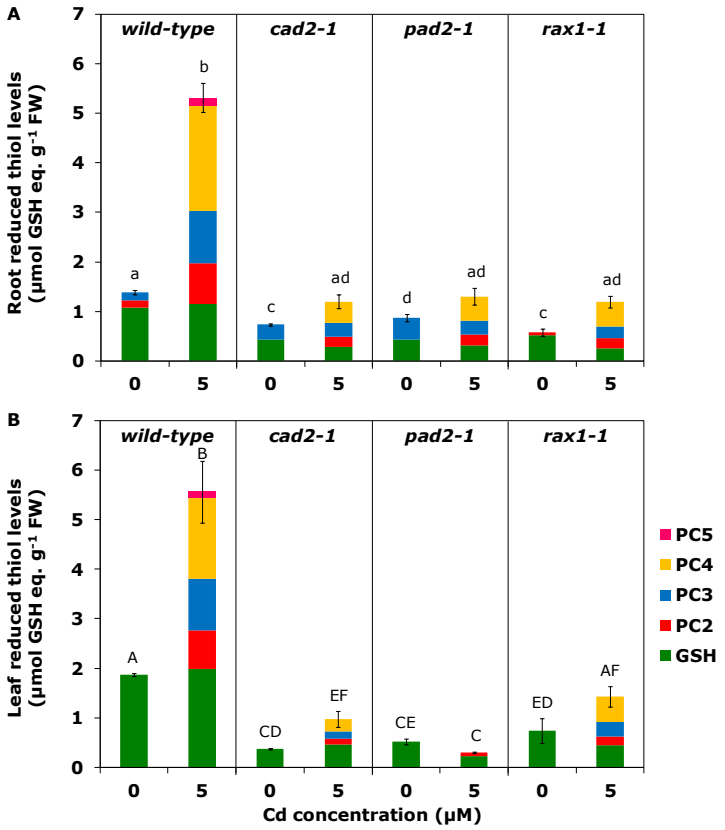


Fig. 6.3 *Profiling the total non-protein thiol content including glutathione [GSH, (■)] and phytochelatins [PC2 (■), PC3 (■), PC4 (■) and PC5 (■)] in four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 h. Thiol content is expressed relative to the wild-type control (= 1.0). Statistical significance in total thiol levels is expressed using lower (roots) and upper (leaves) case letters; n = 4 (two-way ANOVA, P < 0.05).*

Table 6.3 *Concentrations of total ascorbate (AsA) in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h. Data are expressed relative to the control condition of wild-type plants (=100.0%). Statistical significance is expressed using lower (roots) and upper (leaves) case letters; n = 6 (two-way ANOVA within each time point, P < 0.05).*

TOTAL AsA (µmol g^{-1} FW)	**24 h**			**72 h**	
	0 µM Cd	**5 µM Cd**	**genotype**	**0 µM Cd**	**5 µM Cd**
LEAF	1.20 ± 0.18 ^{A}	1.70 ± 0.32 ^{A}	***wild-type***	3.42 ± 0.77 ^{A}	2.22 ± 0.23 ^{A}
	1.89 ± 0.22 ^{A}	2.37 ± 0.40 ^{A}	***cad2-1***	4.04 ± 1.45 ^{A}	3.20 ± 0.60 ^{A}
	2.02 ± 0.87 ^{A}	1.86 ± 0.28 ^{A}	***pad2-1***	5.22 ± 1.31 ^{A}	2.90 ± 0.20 ^{A}
	1.92 ± 0.33 ^{A}	1.48 ± 0.08 ^{A}	***rax1-1***	3.14 ± 0.45 ^{A}	3.54 ± 0.42 ^{A}
ROOT	0.39 ± 0.14 ^{ab}	0.44 ± 0.06 ^{ab}	***wild-type***	6.34 ± 0.40 ^{a}	3.04 ± 1.76 ^{a}
	0.30 ± 0.02 ^{ab}	0.57 ± 0.05 ^{a}	***cad2-1***	5.95 ± 1.80 ^{a}	5.39 ± 1.33 ^{a}
	0.17 ± 0.04 ^{b}	0.50 ± 0.05 ^{a}	***pad2-1***	4.34 ± 1.50 ^{a}	8.76 ± 2.16 ^{a}
	0.33 ± 0.02 ^{ab}	0.63 ± 0.11 ^{a}	***rax1-1***	4.22 ± 1.98 ^{a}	1.68 ± 0.63 ^{a}

6.4.2 Cadmium-induced changes in antioxidant capacity

Although no significant changes were observed, the following trends were apparent in roots (Table 6.3). Exposure to Cd during 24 h resulted in at least twice as high AsA contents in the roots of all mutant plants. After 72 h of Cd exposure, total root AsA levels decreased up to 50% in wild-type and *rax1-1* plants, whereas *cad2-1* plants showed no changes with the control condition and *pad2-1* still possessed twice as high total AsA levels. In leaves of all genotypes, no significant differences on total AsA content were observed upon Cd exposure. Apart from GSH and AsA metabolite levels, two groups of enzymes were analysed at activity level: enzymes involved in the antioxidative defence (GR, CAT, APx, G6PDH and SOD) on one hand and cell wall lignification (GPOD and SPOD) on the other hand. Since no genotype-effects were observed under control conditions (Table 6.4A), activities were expressed relative to their corresponding control condition for each genotype (Table 6.4B). Concerning the antioxidative defence enzymes, changes in CAT and APx activity were minor in all genotypes, although roots of wild-type plants did show a significant five-fold decrease in CAT activity after 24 h exposure to Cd (Table 6.4). Glutathione reductase activity however, showed significant increases after 72 h of Cd exposure, which in roots, were more pronounced in mutant than in wild-type plants. Activity of G6PDH was increased in the leaves of all Cd-exposed genotypes, whereas in roots, only *cad2-1* plants showed significantly increased G6PDH activity after 72 h of Cd exposure. Finally, SOD activity appeared to be constant in wild-type plants with a decreasing trend after 24 h in leaves (Fig. 6.4). In all mutants however, SOD activity was significantly increased in roots after 72 h of exposure, while *cad2-1* plants already responded in this way after 24 h. No significant changes in SOD activity were detected in the leaves, but *cad2-1* and *rax1-1* plants showed increasing trends after 24 h and *pad2-1* plants after 72 h of exposure to Cd. Concerning enzymes involved in lignification, GPOD and SPOD activities were increased in a time-dependent manner in both organs of all genotypes (Table 6.4). In the roots, wild-type plants only showed increasing trends, whereas Cd-exposed mutants showed significant increases in GPOD and/or SPOD activity after 72 h. The leaves demonstrated significantly increased GPOD activity in all genotypes after both 24 and 72 h of Cd exposure. The activity of SPOD increased strongly after 72 h in all genotypes, but after 24 h, only significant increases were found in *pad2-1* and *rax1-1* plants.

Table 6.4 *Enzyme activities in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h. (A) Activities under control conditions are expressed in mU g^{-1} FW and (B) activities upon Cd exposure are expressed relative to the corresponding genotypic control (= 1.0); n = 4 (***** significantly different from the control condition of the corresponding genotype, one-way ANOVA, P < 0.05). Enzymes: glutathione reductase (GR), catalase (CAT), ascorbate peroxidase (APx), glucose-6-phosphate dehydrogenase (G6PDH), guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD).*

0 µM Cd (absolute values in mU g^{-1} FW)

GR		CAT		APX		G6PDH		GPOD		SPOD	
24 h	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**
870.2 ± 41.7	908.3 ± 38.6	22.8 ± 5.0	11.5 ± 0.6	8101 ± 1241	4230 ± 345	66.6 ± 9.3	78.9 ± 13.6	14.1 ± 3.3	18.6 ± 4.3	55.8 ± 7.6	51.6 ± 12.2
938.4 ± 20.5	887.9 ± 52.8	14.7 ± 5.4	18.2 ± 4.2	8565 ± 1632	13612 ± 2328	90.7 ± 8.8	61.6 ± 8.0	10.3 ± 3.1	13.1 ± 0.5	76.9 ± 17.9	43.5 ± 3.2
1074.0 ± 74.9	943.4 ± 74.4	20.1 ± 5.4	17.6 ± 5.6	8037 ± 64	7974 ± 938	93.2 ± 10.9	102.0 ± 11.3	14.3 ± 5.0	21.9 ± 3.6	62.7 ± 15.3	97.2 ± 15.1
1004.3 ± 119.4	880.4 ± 36.7	10.1 ± 1.7	12.0 ± 3.3	8147 ± 1074	8820 ± 1609	66.5 ± 14.3	158.8 ± 60.7	39.7 ± 9.0	52.6 ± 18.1	138.9 ± 30.4	180.9 ± 7.5

GR		CAT		APX		G6PDH		GPOD		SPOD	
24 h	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**
158.8 ± 24.6	111.7 ± 13.4	18.9 ± 4.0	10.2 ± 0.8	11708 ± 1217	10423 ± 1788	203.1 ± 57.6	128.9 ± 27.0	339 ± 119	554 ± 37	844 ± 286	1286 ± 45
125.3 ± 33.4	94.2 ± 15.6	16.0 ± 5.5	7.3 ± 1.5	11796 ± 1921	11060 ± 2175	128.4 ± 49.2	72.6 ± 27.1	529 ± 129	511 ± 205	756 ± 189	1170 ± 542
130.7 ± 10.4	121.6 ± 26.8	15.7 ± 4.5	21.0 ± 4.1	10418 ± 1332	10096 ± 924	53.4 ± 8.5	130.3 ± 40.2	959 ± 142	890 ± 152	1280 ± 204	1257 ± 248
130.2 ± 21.6	112.1 ± 14.6	10.2 ± 3.3	5.1 ± 0.4	12706 ± 1964	10613 ± 743	128.9 ± 35.3	111.3 ± 14.0	731 ± 82	993 ± 166	1506 ± 506	1423 ± 202

5 µM Cd (values relative to the corresponding control = 1.0)

GR		CAT		APX		G6PDH		GPOD		SPOD	
24 h	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**
1.23 ± 0.02*	1.51 ± 0.16*	0.66 ± 0.17	0.89 ± 0.33	1.19 ± 0.13	3.38 ± 0.39*	1.44 ± 0.01*	2.76 ± 0.31*	2.97 ± 0.66*	27.71 ± 10.82*	3.30 ± 1.26	23.16 ± 6.89*
1.18 ± 0.11	1.45 ± 0.18*	1.09 ± 0.13	0.53 ± 0.14	0.75 ± 0.23	1.01 ± 0.22	1.33 ± 0.30	2.79 ± 0.46*	3.74 ± 0.53*	35.72 ± 17.68*	1.35 ± 0.44	23.06 ± 8.80
1.03 ± 0.04	1.45 ± 0.01*	0.61 ± 0.12	0.88 ± 0.26	1.26 ± 0.13	2.39 ± 0.24*	1.55 ± 0.07*	1.98 ± 0.35*	8.24 ± 1.00*	45.80 ± 9.42*	5.84 ± 0.50*	37.06 ± 9.30*
1.13 ± 0.02	1.61 ± 0.03*	2.13 ± 0.44	1.07 ± 0.10	1.25 ± 0.03	1.67 ± 0.22	2.37 ± 0.15*	1.73 ± 0.13	3.82 ± 0.88*	9.35 ± 0.43*	3.31 ± 0.67*	7.32 ± 0.60*

GR		CAT		APX		G6PDH		GPOD		SPOD	
24 h	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**	**24 h**	**72 h**
0.95 ± 0.12	1.51 ± 0.22	0.23 ± 0.02*	0.86 ± 0.19	1.05 ± 0.04	1.32 ± 0.18	0.29 ± 0.02	1.21 ± 0.36	2.08 ± 0.32	2.19 ± 0.35	2.05 ± 0.53	1.57 ± 0.23
1.87 ± 0.23	4.29 ± 0.21*	1.63 ± 0.62	1.52 ± 0.34	0.94 ± 0.15	1.31 ± 0.15	2.47 ± 0.72	3.26 ± 0.79*	1.87 ± 0.28	7.22 ± 0.83*	2.40 ± 0.45*	5.07 ± 0.51*
1.44 ± 0.25	2.49 ± 0.17*	2.49 ± 0.71	0.62 ± 0.11	1.50 ± 0.07*	1.23 ± 0.06	2.95 ± 0.61	0.65 ± 0.20	0.73 ± 0.18	2.45 ± 0.44*	0.74 ± 0.22	1.95 ± 0.97
1.63 ± 0.28	2.86 ± 0.12*	1.20 ± 0.21	3.45 ± 1.42	0.99 ± 0.18	1.54 ± 0.14*	0.73 ± 0.19	2.22 ± 0.65	1.37 ± 0.39	4.04 ± 0.25*	1.18 ± 0.38	4.41 ± 0.30*

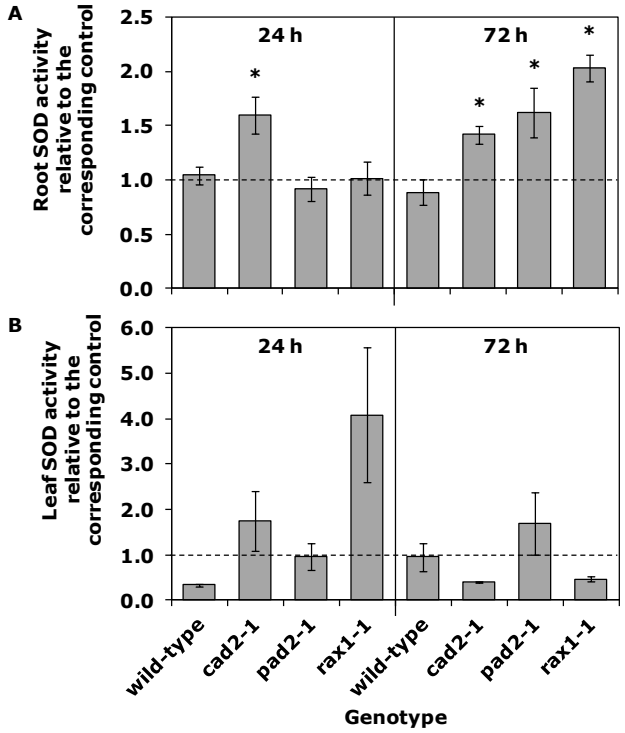


Fig. 6.4 *Superoxide dismutase (SOD) activity relative to the corresponding genotypic control (= 1.0: dashed line) in roots (A) and leaves (B) of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h; n = 4 (***** significantly different from the control condition of the corresponding genotype, one-way ANOVA, P < 0.05).*

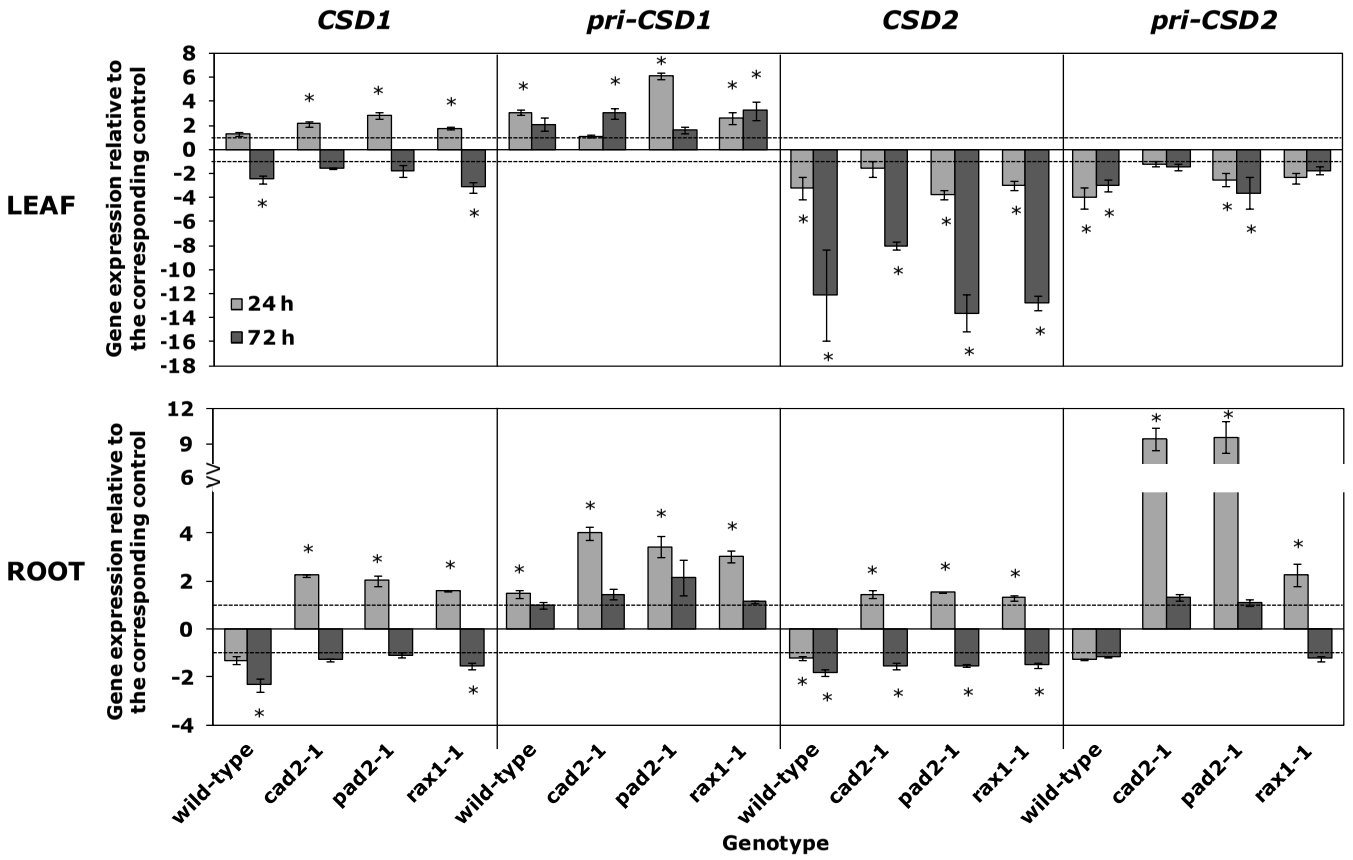
6.4.3 Glutathione deficient plants display an altered transcriptional profile of superoxide dismutase and related regulatory genes

At gene expression level, *MSD1* was unaltered in wild-type plants after 24 h of Cd exposure, whereas a small significant increase was observed in the mutant plants. After 72 h a down-regulation of *MSD1* was noted in the roots of all genotypes, whereas no effect was present in leaves. The expression of *FSD1* was generally induced by Cd exposure in roots after 24 and 72 h and in leaves only after 72 h, except *cad2-1* plants showed decreasing trends in leaves (Table 6.5). Expression of *FSD2/3* however, was generally down-regulated in all genotypes, except for *FSD3* in *cad2-1* plants. This opposite profile of *FSD1* (increase) and *FSD2/3* (decrease), was also observed in the expression of Cu chaperones. The expression of the Cu chaperone for CuZnSOD (*CCS*) was down-regulated, whereas the other chaperone genes, Cu chaperone (*CCH*) and homolog of anti-oxidant 1 (*ATX1*), were up-regulated in all genotypes. In general, primary transcript levels of MIR398 (*pri-MIR398b-c*) were induced in all Cd-exposed genotypes, whereas expression levels of *CSD3* were reduced in both

Table 6.5 *Gene expression patterns relative to the control condition of the corresponding genotype (= 1.00) in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0 or 5 µM $CdSO_4$ for 24 and 72 h; n = 4 (significant up-regulation, ■; significant down-regulation, ■; one-way ANOVA, P < 0.05). Genes: manganese superoxide dismutase 1 (MSD1), iron superoxide dismutase (FSD1-3), copper/zinc superoxide dismutase (CSD1-3), primary microRNA398 transcripts (pri-MIR398b-c), copper chaperone for copper/zinc superoxide dismutase (CCS), copper chaperone (CCH), homolog of anti-oxidant 1 (ATX1).*

	ROOT (gene expression relative to the corresponding control)					**LEAF** (gene expression relative to the corresponding control)				
Time	***wild-type***	***cad2-1***	***pad2-1***	***rax1-1***	**Gene**	***wild-type***	***cad2-1***	***pad2-1***	***rax1-1***	**Time**
24 h	0.95 ± 0.10	1.44 ± 0.14	1.83 ± 0.21	1.20 ± 0.07	***MSD1***	0.95 ± 0.05	1.45 ± 0.02	1.42 ± 0.01	1.32 ± 0.04	**24 h**
72 h	0.71 ± 0.05	0.87 ± 0.04	0.73 ± 0.02	0.75 ± 0.03		0.78 ± 0.13	0.66 ± 0.01	1.01 ± 0.17	0.78 ± 0.01	**72 h**
24 h	29.5 ± 1.85	2.11 ± 0.55	5.90 ± 0.86	2.30 ± 0.40	***FSD1***	3.00 ± 1.03	0.49 ± 0.24	0.86 ± 0.09	0.72 ± 0.10	**24 h**
72 h	26.1 ± 2.11	1.75 ± 0.21	7.70 ± 0.51	1.79 ± 0.26		58.6 ± 12.8	0.49 ± 0.02	5.81 ± 1.02	4.81 ± 0.21	**72 h**
24 h	0.92 ± 0.08	1.06 ± 0.12	0.66 ± 0.03	0.79 ± 0.05	***FSD2***	0.38 ± 0.04	0.59 ± 0.10	0.21 ± 0.03	0.31 ± 0.05	**24 h**
72 h	0.68 ± 0.06	0.80 ± 0.06	0.69 ± 0.05	0.75 ± 0.05		0.55 ± 0.07	0.47 ± 0.11	0.44 ± 0.14	0.47 ± 0.07	**72 h**
24 h	0.81 ± 0.04	1.32 ± 0.12	0.75 ± 0.04	0.65 ± 0.01	***FSD3***	0.24 ± 0.01	1.33 ± 0.25	0.38 ± 0.03	0.31 ± 0.05	**24 h**
72 h	0.70 ± 0.07	1.18 ± 0.04	0.84 ± 0.07	0.59 ± 0.04		0.70 ± 0.05	1.12 ± 0.15	0.81 ± 0.25	0.43 ± 0.05	**72 h**
24 h	0.79 ± 0.09	1.89 ± 0.07	2.30 ± 0.24	1.70 ± 0.04	***CSD1***	1.30 ± 0.17	1.62 ± 0.16	2.51 ± 0.23	1.82 ± 0.15	**24 h**
72 h	0.45 ± 0.05	0.46 ± 0.03	0.75 ± 0.07	0.57 ± 0.05		0.43 ± 0.06	0.47 ± 0.01	0.69 ± 0.19	0.44 ± 0.08	**72 h**
24 h	0.85 ± 0.06	1.29 ± 0.13	1.61 ± 0.01	1.36 ± 0.12	***CSD2***	0.43 ± 0.14	0.71 ± 0.19	0.22 ± 0.03	0.30 ± 0.04	**24 h**
72 h	0.56 ± 0.05	0.46 ± 0.04	0.59 ± 0.03	0.62 ± 0.04		0.12 ± 0.04	0.07 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	**72 h**
24 h	1.17 ± 0.06	1.02 ± 0.09	0.91 ± 0.04	0.63 ± 0.03	***CSD3***	0.41 ± 0.07	1.14 ± 0.21	0.32 ± 0.03	0.38 ± 0.04	**24 h**
72 h	0.84 ± 0.02	0.76 ± 0.03	0.98 ± 0.08	0.66 ± 0.01		0.67 ± 0.05	0.67 ± 0.02	0.69 ± 0.09	0.68 ± 0.02	**72 h**
24 h	2.61 ± 0.24	2.31 ± 0.43	1.86 ± 0.28	1.40 ± 0.24	***pri-MIR398b***	3.16 ± 0.71	3.43 ± 1.31	3.44 ± 1.75	1.52 ± 0.11	**24 h**
72 h	2.89 ± 0.24	1.45 ± 0.19	3.42 ± 0.56	1.48 ± 0.15		18.1 ± 4.12	1.34 ± 0.49	2.88 ± 0.64	1.80 ± 0.11	**72 h**
24 h	5.00 ± 0.75	4.76 ± 1.49	6.35 ± 1.16	2.35 ± 0.50	***pri-MIR398c***	4.36 ± 1.09	5.24 ± 1.93	4.71 ± 2.49	1.99 ± 0.08	**24 h**
72 h	5.18 ± 0.58	2.52 ± 0.39	9.95 ± 1.15	1.73 ± 0.30		32.9 ± 8.61	2.17 ± 0.65	5.48 ± 1.22	7.63 ± 0.26	**72 h**
24 h	0.93 ± 0.10	0.91 ± 0.03	0.76 ± 0.05	0.99 ± 0.05	***CCS***	0.41 ± 0.12	0.47 ± 0.08	0.40 ± 0.03	0.36 ± 0.04	**24 h**
72 h	0.53 ± 0.04	0.56 ± 0.04	0.60 ± 0.09	0.65 ± 0.01		0.11 ± 0.03	0.14 ± 0.03	0.09 ± 0.03	0.08 ± 0.002	**72 h**
24 h	3.52 ± 0.40	3.90 ± 1.03	4.00 ± 0.20	3.38 ± 0.54	***CCH***	1.12 ± 0.09	2.70 ± 0.38	1.92 ± 0.01	1.17 ± 0.11	**24 h**
72 h	3.07 ± 0.22	2.27 ± 0.14	2.76 ± 0.24	3.11 ± 0.07		1.42 ± 0.27	1.31 ± 0.03	1.82 ± 0.30	1.51 ± 0.09	**72 h**
24 h	1.21 ± 0.11	1.57 ± 0.21	1.71 ± 0.20	1.17 ± 0.01	***ATX1***	1.69 ± 0.08	2.08 ± 0.10	1.61 ± 0.12	1.71 ± 0.03	**24 h**
72 h	0.94 ± 0.07	1.26 ± 0.13	1.17 ± 0.08	1.00 ± 0.04		1.31 ± 0.16	1.72 ± 0.17	1.96 ± 0.24	1.27 ± 0.05	**72 h**

Fig. 6.5 *Gene expression patterns of mature (CSD1/2) and primary (pri-CSD1/2) copper/zinc superoxide dismutase transcripts, relative to the control condition of the corresponding genotype (= 1: dashed line) in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 5 µM $CdSO_4$ for 24 (□) and 72 h (■); n = 4 (* significantly different from the control condition of the corresponding genotype, one-way ANOVA, P < 0.05).*



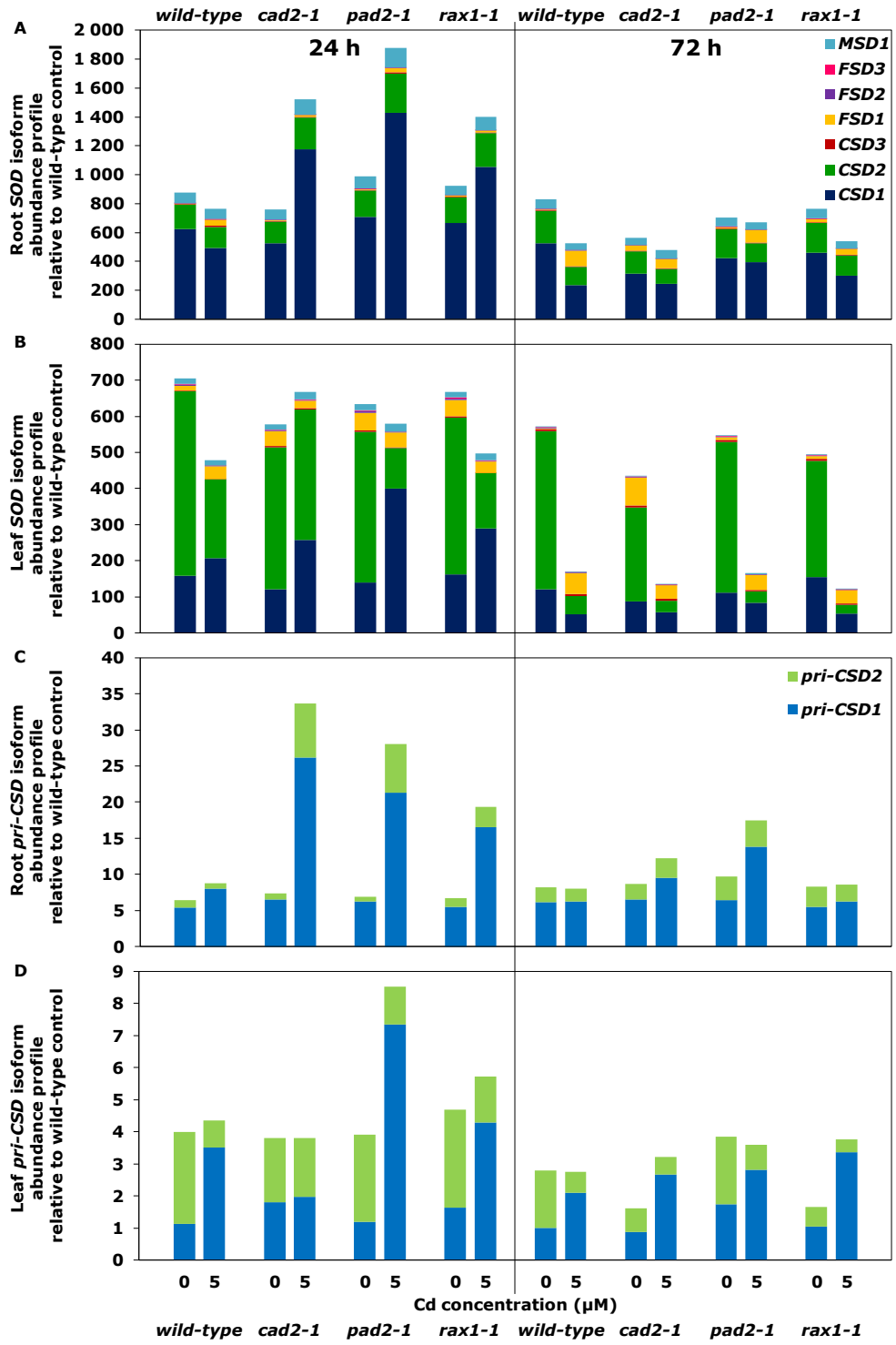

wild-type cad2-1 pad2-1 rax1-1 wild-type cad2-1 pad2-1 rax1-1
A
24 h
72 h
MSD1
FSD3
FSD2
FSD1
CSD3
CSD2
CSD1
Root SOD isoform abundance profile relative to wild-type control
2 000
1 800
1 600
1 400
1 200
1 000
800
600
400
200
0
B
Leaf SOD isoform abundance profile relative to wild-type control
800
700
600
500
400
300
200
100
0
C
pri-CSD2
pri-CSD1
Root pri-CSD isoform abundance profile relative to wild-type control
40
35
30
25
20
15
10
5
0
D
Leaf pri-CSD isoform abundance profile relative to wild-type control
9
8
7
6
5
4
3
2
1
0
0 5 0 5 0 5 0 5 0 5 0 5 0 5 0 5
Cd concentration (µM)
wild-type cad2-1 pad2-1 rax1-1 wild-type cad2-1 pad2-1 rax1-1

Fig. 6.6 *Relative abundance of mature [CSD1 (■), CSD2 (■), CSD3 (■), FSD1 (■), FSD2 (■), FSD3 (■), MSD1 (■)] and primary [pri-CSD1 (■), pri-CSD2 (■)] superoxide dismutase multigene family members in roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 5 µM $CdSO_4$ for 24 and 72 h. Data represent mean abundance relative to the wild-type control and with the abundance of the lowest expressed family member in wild-type set at 1 under the control condition; n = 4. Relative abundance of SOD multigene family members in roots (A) and leaves (B). Relative abundance of pri-CSD multigene family members in roots (C) and leaves (D).*

roots and leaves of all genotypes. In wild-type plants, Cd exposure resulted in *CSD1/2* down-regulation in a time-dependent manner in both organs. In the roots of mutant plants however, significant up-regulation of both genes was observed after 24 h and down-regulation after 72 h of exposure. In the leaves of mutant plants, *CSD1* expression was significantly induced by Cd after 24 h, while decreases or decreasing trends were observed after 72 h. Concerning *CSD2* levels, time-dependent decreases were shown in leaves of mutant plants after exposure to Cd. The primary transcripts of *CSD1* (*pri-CSD1*) on the one hand, were increased or showed increasing trends in all genotypes upon Cd exposure, although all mutants showed significantly stronger inductions in the roots but only after 24 h as compared to wild-type plants. Primary *CSD2* transcripts (*pri-CSD2*) on the other hand, was decreased or showed decreasing trends in Cd-exposed wild-type plants. Mutant *pri-CSD2* levels however, were significantly increased in roots after 24 h of Cd exposure, but were decreased or showed decreasing trends in roots after 72 h and in leaves after both exposure times.

Quantification of the total transcript abundance of all investigated SOD isoforms together, as well as the relative contribution of each member was included in this study. In all genotypes, *FSD2/3* and *CSD3* genes were much less abundant in comparison to *FSD1*, *MSD1* and *CSD1/2* transcripts. In roots, *CSD1* transcripts were most abundant (Fig. 6.6A), while in leaves *CSD2* transcripts were most abundant (Fig. 6.6B), respectively. In contrast to roots of wild-type plants, total *SOD* abundance was increased in roots of mutant plants after 24 h of Cd exposure, which primarily resulted from up-regulated *CSD1* levels. After 72 h, all roots showed decreased abundance in *CSD1/2* and increased *FSD1* transcripts. When *pri-CSD1/2* expression in roots was considered, a strong induction in both primary transcripts was obvious in all mutants after 24 h of exposure, which disappeared over time (Fig. 6.6C). In leaves of wild-type plants, despite increasing *FSD1* levels, strong decreases in total *SOD* transcript

abundance was demonstrated after both 24 and 72 h of Cd exposure, mainly due to down-regulation of *CSD2* (Fig. 6.6B). Leaves of Cd-exposed mutant plants showed the same pattern after 72 h, but unaltered total *SOD* transcript levels after 24 h. Leaf total *pri-CSD* abundance was unaltered in wild-type plants after exposure to Cd because *pri-CSD1* levels were increased and *pri-CSD2* levels were decreased (Fig. 6.6D). In the mutants however, up-regulation of *pri-CSD1* transcripts resulted in Cd-induced increases in total abundance after 24 h in *pad2-1* mutants and after 72 h in *cad2-1* and *rax1-1* mutants.

6.5 Discussion

Glutathione, present at millimolar concentrations in plant cells, is the most abundant non-protein thiol. The level of this metabolite has been shown to increase under various oxidative stress conditions. The accumulation of thiols that has been observed upon Cd exposure can serve multiple functions. First, GSH is a precursor for PCs, the major Cd-chelating peptides (Grill *et al.* 1985; Wójcik & Tukiendorf *et al.* 2004). Secondly, GSH can act directly as an antioxidant or indirectly as a substrate for reducing enzymes, *i.e.* enzymes involved in the AsA-GSH cycle and redoxin cycle (Foyer & Noctor 2011; Jozefczak *et al.* 2012). And finally, changes in GSH redox status and enhanced ROS levels are suggested to be equally important in redox signalling (Noctor & Foyer 1998b; Suzuki *et al.* 2012) and subsequent induction of defence genes (Wingate *et al.* 1988; Ball *et al.* 2004). For these reasons, changes in intracellular GSH status might have important consequences for the cellular responses. To study the effects of decreased GSH levels on plant's response to Cd, three-week-old *A. thaliana* wild-type and GSH deficient mutant plants were exposed to an environmentally realistic Cd concentration of 5 µM (Krznaric *et al.* 2009). Decreased GSH levels were observed in different mutant *GSH1* alleles (Parisy *et al.* 2007). The *rml1* mutant (less than 5% of wild-type GSH contents) was excluded from this study due to its failure to develop a root apical meristem (Vernoux *et al.* 2000). Although the other *GSH1* mutants, containing about 20 to 40% of wild-type GSH levels, showed no or minor developmental phenotypes, different environmental responses are described in comparison to wild-type plants. In the *cad2-1* mutant, lower GSH levels are associated with enhanced Cd sensitivity (Cobbett *et al.* 1998), whereas *pad2-1* and *rax1-1* mutants are more sensitive to biotic and photo-oxidative stress, respectively (Ball *et al.* 2004; Parisy *et al.* 2007).

6.5.1 Glutathione deficiency increases plant's sensitivity towards cadmium

Among the three mutant alleles studied, *rax1-1* mutants possessed the highest amount of GSH (30-40% of the wild-type level), followed by *cad2-1* (20-30%) and *pad2-1* plants (15-30%) (Table 6.2A) as described before (Parisy *et al.* 2007; Sobrino-Plata *et al.* 2014). Previous studies suggest a threshold for minimal GSH levels, below which developmental effects are observed. Severely GSH deficient *rml1* mutants show complete root growth inhibition (Vernoux *et al.* 2000), whereas other GSH deficient mutants with GSH levels above 15% did not show any perturbation of root cell division (Cobbett *et al.* 1998). Therefore, this threshold would lie below the GSH levels in the mutants used in this study (Wójcik & Tukiendorf *et al.* 2011). Additionally, GSH deficient mutants have been indicated to maintain high mitochondrial GSH levels, which are essential for normal plant development and growth (Zechmann *et al.* 2008). These findings are supported by the absence of strong growth disturbances in the mutants under normal conditions in our study (Table 6.1). Besides GSH deficiency, the roots of all mutants showed a more oxidised GSH redox state under control conditions, in comparison to wild-type plants (Table 6.2B). Both GSH levels and its redox state have been suggested to modulate several cellular redox processes at the level of gene expression and protein activity, resulting in enhanced sensitivity towards biotic and abiotic stresses (Chapter 5; Mou *et al.* 2003; Parisy *et al.* 2007).

In agreement with previous studies, Cd exposure resulted in increased Cd levels in both organs, but GSH deficiency was associated with lower translocation to the leaves in comparison to wild-type plants (Fig. 6.1) (Sobrino-Plata *et al.* 2014). The mutant genotypes were characterised by deficiency in GSH (Table 6.2) and hence, PC production (Fig. 6.3). Together with an initially more oxidised environment, these properties resulted in a higher sensitivity to Cd in the mutants than in wild-type plants, which is reflected in significantly decreased fresh weight upon Cd exposure (Table 6.1). Although Cd-induced stress was not reflected at the level of fresh weight in wild-type plants, their roots showed decreased K levels upon 72 h of Cd exposure similar to mutants (Fig. 6.2B), which could be attributed to Cd-induced membrane leakage (Gussarsson & Jensen 1992; Demidchik *et al.* 2010). Additionally, Cd exposure resulted in a

shift towards a more oxidised GSH redox state in roots of wild-type plants (Table 6.2B). As a primary response, plants increased their Cd chelating capacity via PC production (Fig. 6.4) (Jozefczak *et al.* 2014). Elevated PC synthesis has been associated with a transient depletion of GSH levels (Rauser & Meuwly 1995; Jozefczak *et al.* 2014), which was apparent in the roots of wild-type plants and to a lesser extent in the mutants (Table 6.2 and Fig. 6.3). Since PC production was hampered in the GSH deficient mutants and significantly higher Cd levels were present in their roots, the mutants appeared to invest more in lignification of root cell walls to limit the amount of Cd ions entering the cells (Table 6.4B) (Schreiber *et al.* 1999; Cuypers *et al.* 2002). On the other hand, Cd exposure resulted in the activation of several antioxidative defence mechanisms. This included ROS scavenging systems such as SOD (Fig. 6.4 and 6.5) and the AsA-GSH cycle [AsA levels (Table 6.3), APx and GR activity (Table 6.4B)], as well as enzymes generating NADPH as reducing power [*i.e.* G6PDH (Table 6.4B)]. Apart from enhanced lignification upon Cd exposure, activation of SOD and increasing AsA levels were more pronounced in the GSH deficient mutants than in wild-type plants. Together, these findings support that an initially decreased GSH redox state (*i.e.* GSH levels and GSH/GSSG ratio) resulted in elevated Cd sensitivity, which was complemented by the activation of multiple alternative mechanisms (Chapter 4).

6.5.2 Thiols play a regulatory role in copper/zinc superoxide dismutase upon Cd exposure

Wild-type plants confirmed recent findings concerning Cd-induced SOD regulation (Cuypers *et al.* 2011). Although this regulation is consistent with the activation of SPL7 (Yamasaki *et al.* 2007; Nagae *et al.* 2008; Yamasaki *et al.* 2009), it is currently unknown how exposure to Cd triggers these SPL7-dependent responses (Gayomba *et al.* 2013). Posttranslational modification of SPL7 proteins, results in activation and binding of the transcription factor to GTAC motifs in promoters of genes involved in Cu homeostasis (Quinn *et al.* 2000; Kropat *et al.* 2005; Yamasaki *et al.* 2009). On the one hand, a high-affinity uptake system for Cu has been associated with SPL7 activation (Nagae *et al.* 2008; Gayomba *et al.* 2013) and is supported by increasing Cu levels in Cd-exposed roots (Fig. 6.2A). On the other hand, activation of SPL7 is suggested to redistribute Cu to essential Cu-proteins like plastocyanin via up-regulation of

Cu chaperones [*i.e. CCH* (Table 6.5)] and specific microRNAs like MIR398 [*i.e. pri-MIR398b-c* (Table 6.5)] targeting transcripts for dispensable Cu-(binding)proteins [*CCS* (Table 6.5) and *CSD1/2* (Fig. 6.5)] (Weigel *et al.* 2003; Sunkar *et al.* 2006; Yamasaki *et al.* 2009). Although SOD transcript abundance decreased in Cd-exposed wild-type plants (Fig. 6.6), total SOD activity was unaltered (Fig. 6.4) due to Cd-induced *FSD1* up-regulation. Together, these data support the previously described SPL7-dependent responses upon Cd exposure of which the activation mechanism is not fully understood (Cuypers *et al.* 2011; Gayomba *et al.* 2013).

Our previous work showed an opposite regulation of CuZnSOD isoforms in *cad2-1* mutants *versus* wild-type plants after 24 h of exposure to 5 µM Cd (Chapter 4). In order to gain more knowledge on the role of GSH in *CSD* expression under Cd exposure, a more profound study was performed using multiple GSH deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) and exposure times (24 and 72 h). Similar to wild-type plants, SPL7-dependent responses were activated in all Cd-exposed mutants (Fig. 6.2A, Table 6.5) (Gayomba *et al.* 2013). In agreement with our previous findings in *cad2-1* mutants (Chapter 4), both *CSD1* and *CSD2* genes were significantly up-regulated in all three GSH deficient mutants after 24 h of Cd exposure (Fig. 6.5), resulting in significantly increased SOD activities (Fig. 6.4).

Despite different mutations in the regulatory or catalytic function of *GSH1* (Parisy *et al.* 2007), all mutants showed similar responses, excluding mutation-specific effects. Investigating Cd exposure after both 24 and 72 h revealed that the opposite *CSD* response in the mutant *versus* wild-type plants was a fast but transient effect at transcriptional level. Therefore, the increasing root Cu levels after 72 h, although to a greater extent in mutant than in wild-type plants, could not be attributed to this regulation (Fig. 6.2A). Besides SPL7, CuZnSOD regulation has been attributed to two additional factors. First, knock-out studies demonstrated that CCS, encoded by a single gene in *A. thaliana* is responsible for Cu delivery and hence activation of all three CuZnSOD isoforms (Chu *et al.* 2005; Huang *et al.* 2012). However, in our study, *CCS* transcription is down-regulated in all genotypes (Table 6.5) and CuZnSOD activation started at the transcriptional level, excluding a role for CCS. Secondly, a CCS-independent activation of CuZnSOD has been associated with elevated GSH levels (only in

the reduced form) in several organisms (plants, yeast, animals, humans). However, these findings were based on *in vitro* studies and an additional factor seemed to be required, which is still not identified (Ciriolo *et al.* 1990; Hérouart *et al.* 1993; Carroll *et al.* 2004; Jensen & Culotta 2005; Huang *et al.* 2012). The latter mechanism contradicts our *in vivo* findings in the mutants, which associate *CSD* activation with lower instead of higher GSH levels.

In order to further investigate the underlying mechanism of the fast *CSD* induction, primary transcript (*pri-CSD1/2)* levels were included as a measure for transcription rate. Strikingly, the primary transcripts were significantly more up-regulated in the Cd-exposed roots of mutants than wild-type plants after 24 h (Fig. 6.6). These findings indicate elevated transcription rates in the mutants. Such stimulation of the SOD pathway overruling the negative *MIR398* regulation on *CSD* expression in GSH deficient Cd-exposed plants is a new discovery, delivering new insights and opportunities to study the role of GSH in regulating antioxidative defence responses against stress. We support the involvement of an unknown factor and suggest this factor to be a transcriptional activator of *CSD* expression. In this study, two possible hypotheses are presented. First, not only total GSH levels, but changes in both subcellular GSH contents and redox state might trigger *CSD* expression during Cd exposure. Besides mitochondria, the nucleus is known to possess the highest GSH levels under control conditions (Zechmann *et al.* 2008; Zechmann & Müller 2010). Exposure to Cd however, has been associated with a relocation of GSH from nuclei to the cytosol for Cd chelation (Kolb *et al.* 2010). However, specific *in vivo* GSSG detection or labelling in organelles, especially nuclei, is an important missing link to further investigate transcriptional activation by the GSH redox state. Secondly, decreased GSH1 activities in GSH deficient mutants have been associated with cysteine accumulation (Parisy *et al.* 2007). This is in agreement with previous studies, associating elevated cysteine levels with *CSD* up-regulation (Hérouart *et al.* 1993; Youssefian *et al.* 2001). Interestingly, Hérouart and co-workers (1993) performed promoter studies in protoplasts concerning the cytosolic *CSD* gene of *Nicotiana plumbaginifolia*. They observed a marked induction of this gene by several antioxidant thiol components (*i.e.* GSH, cysteine, N-acetyl cysteine and dithiothreitol), whereas the effect was lost with pro-oxidants (H_2O_2 and $O_2^{\bullet-}$) or oxidised thiols (GSSG and cystine). When cells were pre-treated with the GSH1

inhibitor buthionine sulphoximine (BSO), cysteine was still able to induce *CSD* expression. Together, our findings point towards an interesting role for cysteine in the induction of plant *CSD* genes upon Cd exposure. The question remains how cysteine acts on the transcriptional mechanism and whether this role is related to its total concentration and/or reduction-oxidation state.

6.5.3 Conclusion

Investigating GSH deficient mutants revealed strong Cd-induced transcriptional activation of CuZnSOD transcripts, overruling the MIR398-regulated repression. This study suggests that the cysteine redox state (concentration and/or reduced-oxidised ratio) contributes to the transcription rate of *CSD1* and *CSD2* genes upon Cd exposure. However, further analysis of the factors that interact with the CuZnSOD promoter region is essential to identify the transcriptional activator responsible for this regulation.

Appendices

Table A.6.1 *List of primers used in Reverse Transcription quantitative PCR (RTqPCR). Exon-Exon-junction (E-E-jn), untranslated region (UTR).*

Gene	TAIR: locus	Annotation	Primer sequences	Exon location	Amplicon size	Primer efficiency
ATX1	*AT1G66240*	Homolog anti-oxidant 1	FW: TCACAGCTCCAACACATCCCT REV: CGATGGCGACTGCTCTCACT	Exon 2	91 bp	98.2 %
CCH	*AT3G56240*	Cu chaperone	FW: CTTTCACTGTCACCTTTTGCTCCT REV: TGCGTTGGTGCCGTCAATA	Exon 1	91 bp	91.8 %
CCS	*AT1G12520*	Cu chaperone for superoxide dismutase	FW: TTCACAGCATTAACACAACCCTCA REV: CAAGCCTTGTCGGTGGTTGA	Exon 4	91 bp	92.3 %
CSD1	*AT1G08830*	CuZn superoxide dismutase1	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5	102 bp	93.8 %
CSD2	*AT2G28190*	CuZn superoxide dismutase2	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6	101 bp	93.9 %
CSD3	*AT5G18100*	CuZn superoxide dismutase3	FW: GTTGTTGTGCATGCGGATCC REV: CACATCCAACTCTCGAGCCTG	Exon 5	91 bp	91.6 %
FSD1	*AT4G25100*	Fe superoxide dismutase1	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4	101 bp	88.8 %
FSD2	*AT5G51100*	Fe superoxide dismutase2	FW: TTGGAAAGGTTCAAGTCGGCT REV: CATTTGCAACGTCAAGTCTATTCG	Exon 5	91 bp	92.8 %
FSD3	*AT5G23310*	Fe superoxide dismutase3	FW: AACGGGAATCCTTTACCCGA REV: TGTCTCCACCACCAGGTTGC	Exon 3	91 bp	88.6 %
MSD1	*AT3G10920*	Mn superoxide dismutase1	FW: ATGTTTGGGAGCACGCCTAC REV: AACCTCGCTTGCATATTTCCA	Exon 5	101 bp	89.4 %
pri-CSD1	*AT1G08830*	Primary CuZn super-oxide dismutase1	FW: ACTGTTGGAGATGATGGTATGCCT REV: GAGAGAGTAGCGAAATTTGATGCAA	E3-E4-jn	93 bp	91.2 %
pri-CSD2	*AT2G28190*	Primary CuZn super-oxide dismutase2	FW: TCACTATGACTTAGGCTGCGATTG REV: GATGGTCCGAATTTGCGATTAA	Intron 7	102 bp	82.7 %
pri-MIR398b	*AT5G14545*	Primary microRNA398b	FW: AGTAATCAACGGCTGTAATGACGCTAC REV: TGACCTGAGAACACATGAAAACGAGAG	Exon 1	67 bp	85.3 %
pri-MIR398c	*AT5G14565*	Primary microRNA398c	FW: TCGAAACTCAAACTGTAACAGTCC REV: ATTTGGTAAATGAATAGAAGCCACGGGCCACG	Exon 2	241 bp	104.2 %
PPR	*AT5G55840*	Pentatricopeptide repeat superfamily	FW: AAGACAGTGAAGGTGCAACCTTACT REV: GTTTTTGAGTTGTATTTGTCAGAGAAAG	3'UTR	59 bp	81.5 %
SAND	*AT2G28390*	SAND family	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13	61 bp	107.8 %
Tip41-like	*AT4G34270*	Tip41-like	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn	61 bp	91.2%
UBC	*AT5G25760*	Ubiquitin conjugating enzvme21	FW: CTGCGACTCAGGGAATCTTCTAA REV: TTGTGCCATTGAATTGAACCC	E3-E4-jn	61 bp	98.7%

Table A.6.2 *Reverse Transcription quantitative PCR (RTqPCR) parameters according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines derived from Bustin et al. 2009.*

Sample/Template	
Source	*Arabidopsis thaliana* roots or shoots in a hydroponic culture
Method of preservation	harvest in liquid N_2 then stored at -80°C
Storage time	one week
Handling	frozen
Extraction method	columns: RNAqueous Total RNA Isolation Kit* (Ambion, Lennik, Belgium)
RNA: DNA-free	TURBO DNA-free Kit* (Ambion, Lennik, Belgium) intron-spanning primers and verification of single peak on melt curves
Concentration	NanoDrop®: ND-1000 Spectrofotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Microfluidics: Bioanalyzer* (Agilent Technologies, Waldbronn, Duitsland) for a representative subset of the samples
Assay optimisation/validation	
Accession number	see Table A.6.1
Amplicon details	exon location & amplicon size: see Table A.6.1
Primer sequence	see Table A.6.1
In silico	Primer-BLAST
Empirical	primer concentration (300 nM) annealing temperature (60°C)
Priming conditions	random hexamer priming
PCR efficiency	dilution curves (slope, y-intercept & r^2, see Table A.6.1)
Linear dynamic range	samples are within the efficiency curve
RT/PCR	
Protocols	TURBO DNA-free Kit* (Ambion) Fast SYBR Green* (Applied Biosystems, Paisley, UK) see Materials and Methods
Reagents	see Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software, version 1.4 (Applied Biosystems, Lennik, Belgium, 2001-2006)
Statistical justification	4 biological replicates, one-way ANOVA and Tukey post-hoc adjustment for multiple comparison
Normalisation	3 reference genes selected using geNorm, version 3.5 (Centre for Medical Genetics, Ghent, Belgium, 2001-7)

*All procedures were performed according to the manufacturer's protocol.

Chapter 7

The fast phytochelatin-mediated response in cadmium-exposed *Arabidopsis thaliana* plants attenuates over time

Research article – in preparation for Plant and Soil

7.1 Abstract

This long-term exposure study determined the impact of cadmium (Cd) on plant growth and leaf morphology. Dose-dependent disturbances were observed at the level of vegetative growth. However, the emergence of inflorescences in all conditions, confirmed that exposure of *Arabidopsis thaliana* to 5 and 10 µM Cd does not cause lethality. In order to investigate the well-known phytochelatin (PC)-mediated Cd tolerance, PC production was followed over time. Plant-internal PC to Cd molar ratios (SH/Cd) showed a time-related increase until three days of Cd exposure, after which the ratios appeared to decrease over time. The latter was suggested to result from decreasing free cytosolic Cd concentrations, which directly affect phytochelatin synthase (PCS) activity. Glutathione (GSH) deficient mutants included in this study showed that despite their increased Cd sensitivity, the mutants survived the Cd-induced toxicity. Together, these data support that after a fast PC response, including both chelation and compartmentalization of Cd, plants activate alternative mechanisms that complement this early Cd detoxification processes.

7.2 Introduction

Toxic metals occur naturally in soils but are often not easily bioavailable. Anthropogenic activities are responsible for the release of metals like cadmium (Cd) in biologically available forms, which may damage the entire ecosystem (Järup & Akesson 2009). Although Cd is a non-essential element, it is taken up in plants via competition with other essential divalent cations for their respective transport systems. Both zinc (Zn) specific ZIP transporters and calcium (Ca) channels have been reported to transport Cd. Once inside plant cells, Cd is bound to sulfur (S)- (Van Belleghem *et al.* 2007) or oxygen (O)-based ligands (Koren *et al.* 2013). Cadmium ions primarily bind to S-ligands due to their higher affinity for thiol (SH) groups than hydroxyl (OH) or carboxyl (COOH) groups (Clemens 2006). A major SH-containing compound involved in Cd chelation is phytochelatin (PC), the synthesis of which is strongly induced upon exposure to Cd. Formation of PC-Cd complexes in the cytosol is followed by sequestration of Cd ions to metabolically less active tissues (*i.e.* tracheids, veins, epidermis) and structures (*i.e.* vacuoles, cell walls), which prevents them to

interfere with vital processes such as photosynthesis and respiration (Clemens 2006; Jozefczak *et al.* 2012). In the acidic vacuole, the PC-Cd complexes have been proposed to dissociate. Subsequently, Cd might be complexed by vacuolar O-ligands of organic acids (e.g. citrate, oxalate, malate), while PCs are degraded by vacuolar hydrolases and/or return to the cytosol (Sanita di Toppi & Gabbrielli 1999).

The basic structure of PCs is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ in which *n* ranges from 2 to 11. Phytochelatin synthase (PCS) enzymatically synthesises PCs from glutathione (GSH) or GSH-related compounds through transpeptidation of the *γ*-Glu-Cys moiety of GSH either to another GSH or to a growing PC peptide. This implies the importance of GSH as a precursor of PC, which is synthesised in two ATP-dependent steps: first cysteine and glutamate are bound by *γ*-glutamylcysteine synthetase (GSH1), followed by the addition of glycine to this *γ*-glutamylcysteine (*γ*-EC) dipeptide by glutathione synthetase (GSH2). Since each GSH biosynthesis gene is encoded by only one identified gene in *Arabidopsis thaliana*, GSH deficiency is easily obtained for biochemical and molecular studies. On the one hand, buthionine sulfoximine (BSO) has been used to prevent GSH synthesis via inhibition of the rate-limiting step, *i.e.* γ-EC synthesis by GSH1 function. On the other hand, multiple *A. thaliana* plants are readily available in databases with different mutations in the *GSH1* gene (*cad2-1*, *pad2-1*, *rax1-1*). Numerous studies provide ample evidence that PC-based Cd sequestration is essential in Cd detoxification in non-accumulator plants like *A. thaliana* (Cobbett *et al.* 1998; Schat *et al.* 2002; Wójcik & Tukiendorf *et al.* 2011; Sobrino-Plata *et al.* 2014).

In several studies, over-expression of enzymes involved in S assimilation, GSH and/or PC synthesis was performed in order to study Cd responses. Although increased Cd tolerance was expected, contrasting results ranging from enhanced tolerance to hypersensitivity were observed (Zhu *et al.* 1999a; Lee *et al.* 2003a; Dominguez-Solis *et al.* 2004; Li *et al.* 2004). Additionally, studies in Cd hyperaccumulators and tolerant plants like *Arabidopsis halleri* and *Thlaspi caerulescens* revealed that Cd resistance did not rely on PC synthesis in these species (de Knecht *et al.* 1995; Schat *et al.* 2002; Huguet *et al.* 2012). In response to these observations, the current study investigated the accumulation of Cd and PCs in *A. thaliana* during a period of three weeks upon exposure to

environmentally realistic Cd concentrations (Krznaric *et al.* 2009). Moreover, effects of long-term Cd exposure on GSH deficient mutants were included in order to examine the necessity of Cd-induced PC production for plant survival.

7.3 Materials and methods

7.3.1 Plant material, growth conditions and harvest

Wild-type (Lehle seeds, Round Rock TX, USA) and GSH-deficient *Arabidopsis thaliana* (Columbia ecotype) seedlings mutated in *GSH1* (*AT4G23100*) were cultivated. The Cd-sensitive *cad2-1* mutant (deletion of P237, K238 and V239L) was donated by Dr. Christopher Cobbett (Melbourne University, Australia; Cobbett *et al.* 1998). The phytoalexin-deficient *pad2-1* mutant (substitution of S298N) was obtained from the European *Arabidopsis* Stock Centre (uNASC ID: N3804, UK; Parisy *et al.* 2007). The regulator of ascorbate peroxidase 2 *rax1-1* mutant (substitution of R228K) was a gift of Prof. Dr. Phil Mullineaux (University of Essex, England; Ball *et al.* 2004). All seeds were surface sterilised and grown under the following conditions: 12 h photoperiod; 22/18°C day/night regime; 170 µmol m^{-2} s^{-1} light at the leaf level; and 65% relative humidity (Chapter 3 and 4).

At the age of three weeks, plants were exposed to 0, 5 or 10 µM Cd supplied as $CdSO_4$ to the roots in the Hoagland solution (Chapter 3). The hydroponic medium was changed once every week to refresh the medium, with or without Cd, to ensure and maintain sufficient nutrient availability (Keunen *et al.* 2011b). Every three to four days, rosette diameter was measured and samples were harvested after 0, 1, 3, 7, 14 and 21 days. The aerial part and roots were separated and dried prior to element determination or snap frozen in liquid nitrogen, then stored at -80°C, prior to thiol extraction.

To analyse root growth, plants were cultivated on square Petri dishes (120 mm x 120 mm) as described before (Chapter 4; Remans *et al.* 2012a). The medium consisted of a 50-fold dilution of the Gamborg B5 medium (Gamborg *et al.* 1968; Zhang & Forde 1998), supplemented by 0.05% (w/v) MES, 0.5% (w/v) sucrose, 1% (w/v) agar. One week after sowing, plants were transferred to fresh medium, without sucrose, containing the following range of Cd concentrations: 0, 1, 2, 5, 7.5 and 10 µM. Root growth was analysed one week after exposure using Optimas (Optimas Corporation, version 6.1, UK).

7.3.2 Element determination

At harvest, roots were washed with ice-cold 10 mM $Pb(NO_3)_2$ (15 min, 4°C) and rinsed with distilled water. Leaves were washed in distilled water. The fresh material was dried at 60°C during two weeks, weighed, and digested in 70-71% HNO_3 using a heat block. Element concentrations were measured via inductively coupled plasma-optical emission spectrometry (ICP-OES 710, Agilent Technologies, Australia).

7.3.3 Analysis of non-protein thiols by HPLC method

Glutathione and PC contents were determined using High-Performance Liquid Chromatography (HPLC) as described earlier (Wójcik & Tukiendorf *et al.* 2005). In brief, frozen root and leaf samples were ground in an ice-cooled mortar with a double volume of 0.1 M HCl. The supernatant resulting after three centrifugation steps (14 000 rpm, 4°C, 5 min) was used in a Beckman chromatograph (126/166 model) with a Supelco precolumn (4.6 mm x 10 mm) and column (4.6 mm x 250 mm), both filled with Ultrasphere C18. The peptides (100 µl) were separated in a linear gradient (0 – 20%) of acetonitrile in 0.05% trifluoroacetic acid and were subjected to a post-column reaction with 200 µM 5.5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.05 M potassium-phosphate buffer (pH 7.6). A Beckman detector (model 166) measured the absorbance at 405 nm and the chromatograms were analysed using Karat 7.0 software from Beckman.

7.3.4 Statistical analysis

All data were analysed with general linear two-way ANOVA models (Verbeke & Molenberghs 2000). Normal distribution and homoscedasticity were tested using the Shapiro-Wilk and Bartlett test, respectively. Tukey post-hoc adjustment was used to correct for multiple comparison. Logarithmic transformations were applied when necessary to approximate normality. The dataset containing the rosette diameters was analysed with one-way ANOVA. All statistical analyses were performed using R (the R foundation of statistical computing, version 2.15.1). Data are mean values ± standard error (SE) and significance was set at the 5% level ($P < 0.05$).

7.4 Results

7.4.1 Cadmium disturbs vegetative growth in Arabidopsis thaliana wild-type plants

Fresh weight and rosette growth were studied as characteristics of vegetative growth in 3-week-old wild-type plants subsequently exposed to Cd during three weeks or grown under control conditions. In this kinetic set-up, root and leaf fresh weight showed a strong increase over time under control conditions (Table 7.1). After three days of Cd exposure however, this process was affected in a dose-dependent manner. The lowest dose (5 µM Cd) resulted in significant growth retardation in both organs, while exposure to the highest dose (10 µM Cd) caused growth arrest in roots, whereas leaf biomass was still increasing but to a minor extent as compared to controls and 5 µM Cd-exposed plants. Both root and leaf dry weight percentages were constant over time in unexposed plants (Fig. 7.1). Exposure to the highest dose resulted in a time-dependent increase in percentage dry weight. The lowest dose however, showed an increasing trend, which was only significant at two weeks of exposure. Concerning rosette diameter, control plants showed significant increases after 3, 7 and 14 days, followed by a plateau phase (Fig. 7.2). Plants exposed to 5 µM Cd demonstrated a reduced rosette expansion in comparison to unexposed plants, but with significant increased rosette diameter after 10 and 17 days, before reaching a plateau phase at about half the diameter of control plants. The highest dose however, resulted in rosette growth arrest. Additionally, control conditions revealed vital green plants during the entire experiment, while Cd exposure caused a time- and dose-dependent increase in chlorosis (Fig. 7.3). However, all plants began their regenerative process after three weeks of Cd exposure (*i.e.* six weeks of growth), indicated by the development of the inflorescence meristem.

Table 7.1 *Fresh weight (mg FW plant^{-1}) of roots and leaves of Arabidopsis thaliana exposed to 0, 5 or 10 µM $CdSO_4$ in hydroponics for different time periods (0, 1, 3, 7, 14, 21 days). Statistical significance is expressed using lower (roots) and upper case letters (leaves); n = 14 (two-way ANOVA, P < 0.05).*

LEAVES (mg FW plant^{-1})			
days	**0 µM Cd**	**5 µM Cd**	**10 µM Cd**
0	46.91 ± 2.80 ^{A}	46.95 ± 3.03 ^{A}	45.70 ± 2.88 ^{A}
1	55.35 ± 3.55 ^{B}	50.15 ± 2.35 ^{AB}	52.55 ± 3.01 ^{AB}
3	106.13 ± 6.37 ^{CD}	64.78 ± 4.20 ^{B}	51.93 ± 2.61 ^{B}
7	213.99 ± 12.98 ^{E}	113.22 ± 5.47 ^{D}	68.32 ± 5.93 ^{B}
14	650.04 ± 51.66 ^{F}	295.13 ± 14.50 ^{G}	102.40 ± 5.57 ^{D}
21	974.92 ± 56.96 ^{H}	455.27 ± 23.88 ^{I}	88.46 ± 4.69 ^{D}
ROOTS (mg FW plant^{-1})			
days	**0 µM Cd**	**5 µM Cd**	**10 µM Cd**
0	15.05 ± 0.55 ^{a}	15.35 ± 0.47 ^{a}	16.08 ± 0.77 ^{a}
1	23.20 ± 1.13 ^{bcd}	19.34 ± 1.01 ^{abc}	19.79 ± 1.17 ^{abc}
3	49.21 ± 4.11 ^{e}	25.16 ± 1.08 ^{abc}	17.09 ± 1.09 ^{cg}
7	88.21 ± 6.93 ^{f}	31.08 ± 1.81 ^{d}	13.21 ± 0.98 ^{g}
14	251.77 ± 29.14 ^{h}	65.38 ± 3.25 ^{de}	13.16 ± 0.93 ^{g}
21	396.08 ± 45.00 ^{i}	90.96 ± 7.59 ^{f}	14.26 ± 1.21 ^{cg}

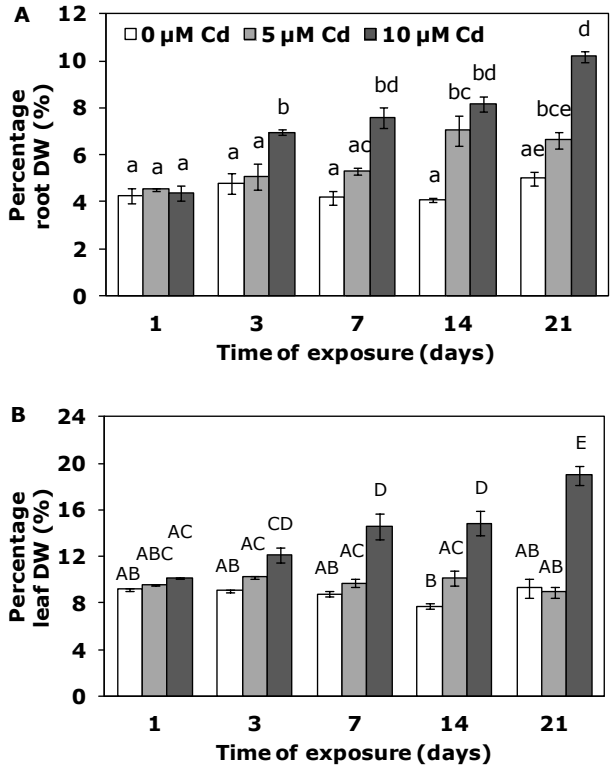


Fig. 7.1 *(A) Root and (B) leaf percentage dry weight (DW) of Arabidopsis thaliana exposed to 0 (□), 5 (▒) or 10 (■) µM $CdSO_4$ in hydroponics for different time periods (0, 1, 3, 7, 14, 21 days). Statistical significance is expressed using lower (roots) and upper case letters (leaves); n = 4 (two-way ANOVA, P < 0.05).*

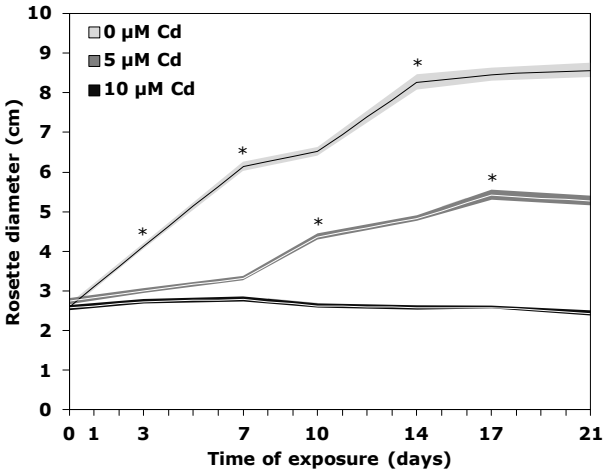


Fig. 7.2 *Rosette diameter (cm) of Arabidopsis thaliana exposed to 0 (□), 5 (▒) or 10 (■) µM $CdSO_4$ in hydroponics for different time periods (0, 1, 3, 7, 10, 14, 17, 21 days). Statistically significant increases over time are indicated by *; n = 14 (one-way ANOVA, P < 0.05).*

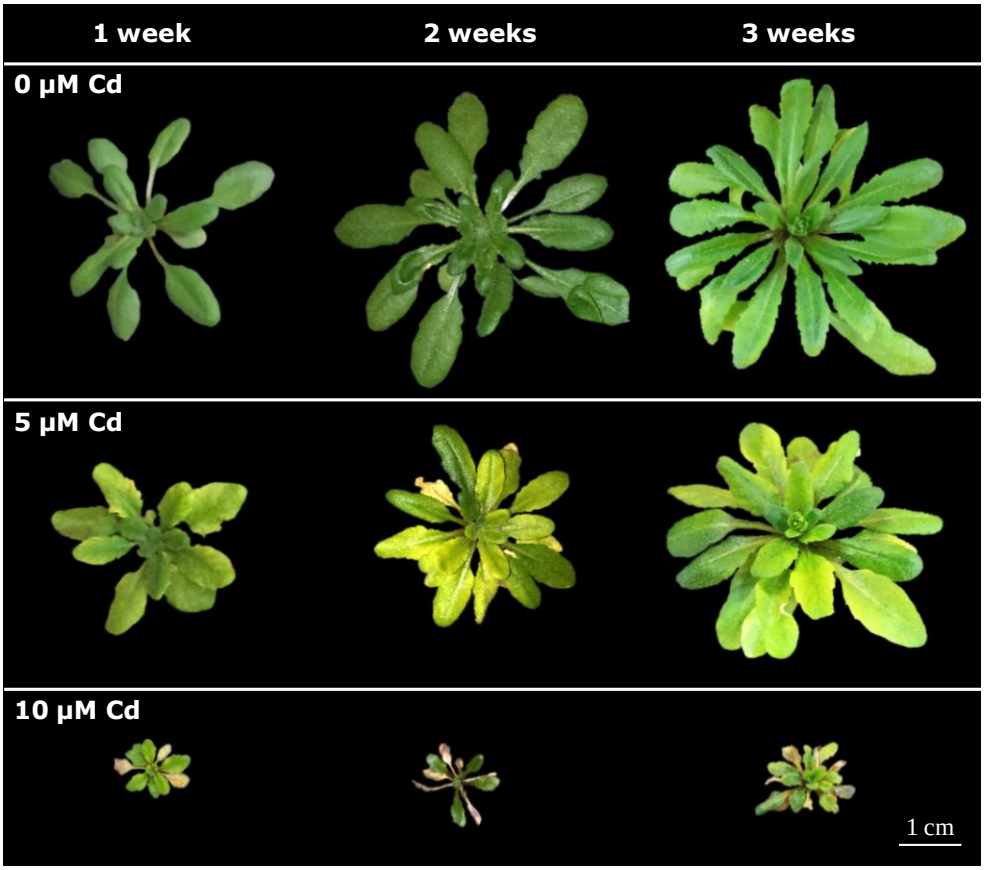


Fig. 7.3 *Representative pictures of the rosette appearance of Arabidopsis thaliana exposed to 0, 5 or 10 µM $CdSO_4$ in hydroponics during 1, 2 and 3 weeks; n = 14.*

7.4.2 Element uptake and distribution in control and Cd-exposed Arabidopsis plants

In roots of Cd-exposed plants, a time- and dose-dependent increase in Cd content was observed (Fig. 7.4A). In leaves however, Cd levels were lower and reached a plateau after one week (Fig. 7.4B). Concerning the essential elements, root concentrations of Cu, Fe, Zn and Ca were increased, whereas Mg and K decreased in a time-dependent manner in plants exposed to 10 µM Cd (Table 7.2). Except for Cu concentrations, the other elements were less affected after exposure of plants to 5 µM Cd. In the leaves, Cu, Ca, K and Mg levels were significantly decreased when exposed to the highest dose, whereas no effects on these nutrients could be observed in 5 µM Cd-exposed plants (Table 7.2).

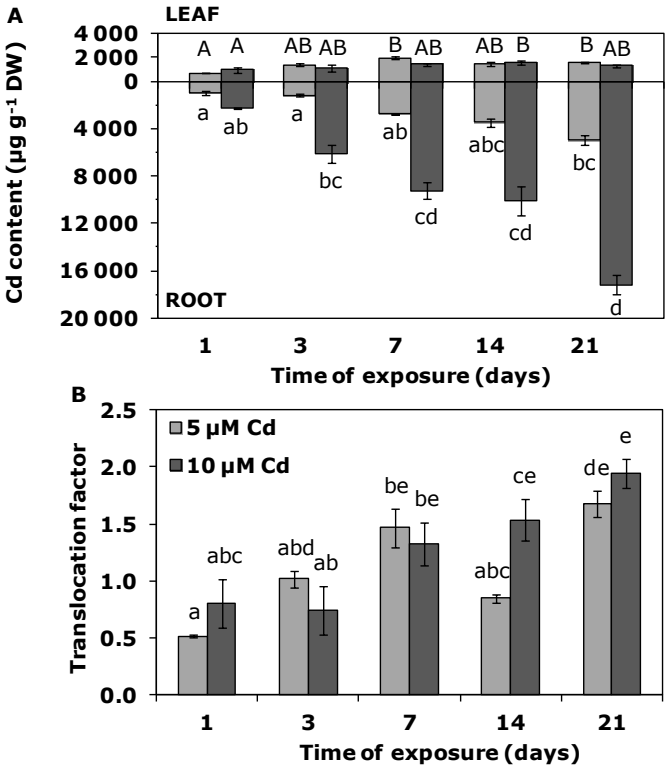


Fig. 7.4 *(A) Root and leaf Cd concentrations (µg g^{-1} DW) and (B) the corresponding translocation factor from roots to shoots of Arabidopsis thaliana exposed to 5 (□) or 10 (■) µM $CdSO_4$ in hydroponics for different time periods (1, 3, 7, 14, 21 days). Note the different scales on the y-axes between both organs. Statistical significance is expressed using lower (roots and translocation factor) and upper (leaves) case letters; n = 4 (two-way ANOVA, P < 0.05).*

Table 7.2 *Element concentrations of roots and leaves relative to the control condition (=100%) of Arabidopsis thaliana exposed to 5 or 10 µM $CdSO_4$ in hydroponics for different time periods (1, 3, 7, 14, 21 days). Statistically significant differences between Cd-exposed and unexposed samples are expressed using *; additional significant differences between both Cd-exposed conditions is expressed using $^+$; n = 4 (two-way ANOVA, P < 0.05).*

	ROOT		relative to	LEAF		
days	**5 µM Cd**	**10 µM Cd**	**0 µM Cd (%)**	**5 µM Cd**	**10 µM Cd**	**days**
1	127.75 ± 13.11	128.00 ± 13.24		86.12 ± 4.01	76.38 ± 1.66*$^+$	**1**
3	267.28 ± 16.61*	367.59 ± 27.25*		83.83 ± 4.07	62.86 ± 3.65*	**3**
7	465.80 ± 12.84*	722.55 ± 45.62*$^+$	**Cu**	86.62 ± 1.46	51.56 ± 2.91*$^+$	**7**
14	394.08 ± 20.89*	1 041.80 ± 156.53*$^+$		98.55 ± 8.91	67.29 ± 2.27	**14**
21	412.60 ± 15.46*	1 457.73 ± 113.75*$^+$		88.52 ± 4.81	57.50 ± 3.95*	**21**
1	99.60 ± 3.76	119.53 ± 11.30		80.35 ± 11.37	87.56 ± 17.88	**1**
3	108.65 ± 7.61	213.49 ± 34.93*$^+$		135.41 ± 54.77	78.00 ± 14.68	**3**
7	200.55 ± 7.96*	399.57 ± 33.82*	**Fe**	37.15 ± 3.56	77.98 ± 10.73	**7**
14	203.91 ± 23.70	631.54 ± 124.82*$^+$		91.44 ± 18.77	274.79 ± 79.11	**14**
21	183.87 ± 8.46*	670.52 ± 17.40*$^+$		28.04 ± 0.94	78.98 ± 23.32	**21**
1	59.67 ± 11.27	58.77 ± 10.39		72.25 ± 7.76	72.65 ± 6.26	**1**
3	110.39 ± 11.05	192.35 ± 41.56		86.17 ± 12.37	74.77 ± 14.54	**3**
7	158.34 ± 18.24	279.92 ± 11.25*	**Zn**	95.51 ± 6.34	68.17 ± 9.28	**7**
14	227.22 ± 39.98*	341.65 ± 34.92*		112.91 ± 6.28	71.55 ± 5.95	**14**
21	188.37 ± 4.41	419.19 ± 12.76*$^+$		127.86 ± 6.95	66.90 ± 9.83	**21**
1	103.92 ± 3.43	112.14 ± 2.69		100.19 ± 0.93	98.61 ± 2.16	**1**
3	116.25 ± 5.66	190.47 ± 9.60*$^+$		93.26 ± 2.83	75.40 ± 5.06*	**3**
7	127.86 ± 5.45	153.97 ± 15.56*	**Ca**	85.03 ± 4.00	57.01 ± 2.82*$^+$	**7**
14	218.50 ± 10.63*	189.33 ± 6.77*		85.27 ± 7.11	60.54 ± 2.17*	**14**
21	141.90 ± 6.30*	157.26 ± 10.93*		101.10 ± 2.55	64.71 ± 4.21*$^+$	**21**
1	90.29 ± 0.69	82.94 ± 3.48		104.51 ± 1.58	95.66 ± 3.01	**1**
3	86.73 ± 5.63	100.95 ± 11.98		101.36 ± 3.85	75.59 ± 6.48	**3**
7	88.26 ± 5.81	73.30 ± 3.63	**Mg**	92.26 ± 4.82	49.99 ± 1.15*$^+$	**7**
14	94.87 ± 10.91	64.61 ± 2.13*		74.60 ± 11.99	47.45 ± 2.72*	**14**
21	60.68 ± 2.38*	40.83 ± 1.84*$^+$		88.83 ± 6.81	48.13 ± 2.35*	**21**
1	108.55 ± 2.87	100.54 ± 7.60		111.06 ± 1.38	101.52 ± 0.97	**1**
3	88.42 ± 6.47	59.76 ± 6.00*$^+$		89.75 ± 3.32	76.72 ± 3.82	**3**
7	81.86 ± 3.57	49.98 ± 4.32*$^+$	**K**	89.29 ± 2.91	58.49 ± 1.61*	**7**
14	72.10 ± 12.46	57.49 ± 9.87*		97.67 ± 13.66	64.84 ± 2.31	**14**
21	114.52 ± 2.34	37.87 ± 1.86*$^+$		103.70 ± 8.03	51.84 ± 3.44*	**21**

7.4.3 Differential profile of thiol production over time in roots and leaves

In order to investigate Cd chelation, GSH and PC production were measured over time. In control plants, relatively stable GSH levels but no PCs were detected. In roots, a time- and dose-dependent increase in total PC levels was observed, which reached a plateau after three days of Cd exposure (Fig. 7.5A). In leaves however, PC concentrations increased in a time- and dose-dependent manner during the initial three days, followed by a time-dependent decrease during the subsequent weeks (Fig. 7.5B). Also the average ratio of GSH/PC and Cd [SH (mol)/Cd (mol)] was calculated (Fig. 7.6). In both organs, SH/Cd increased in a time-dependent manner during the first three days and subsequently, a time-dependent decrease was observed.

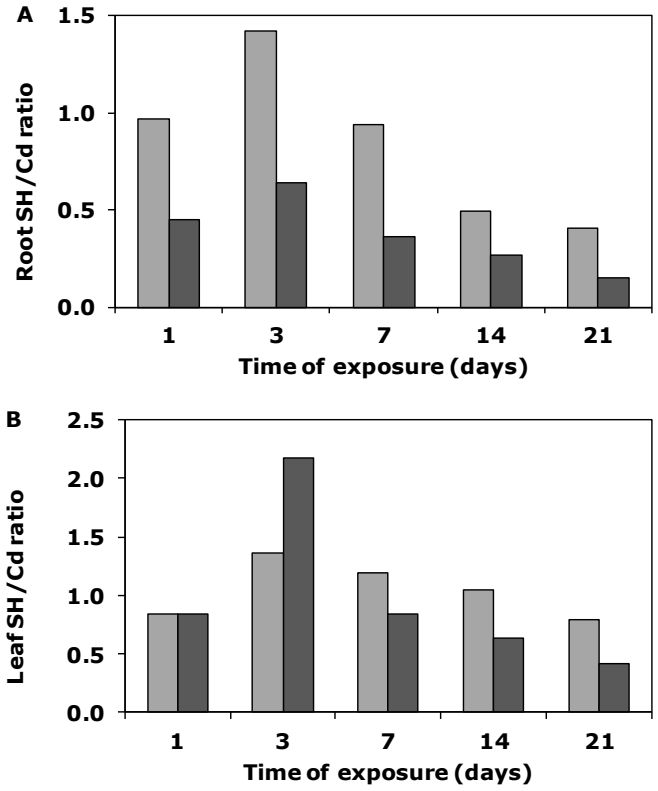


Fig. 7.6 *Average glutathione and phytochelatin to Cd molar ratios (SH/Cd) in roots (A) and leaves (B) of Arabidopsis thaliana exposed to 5 (□) or 10 (■) µM $CdSO_4$ in hydroponics for different time periods (1, 3, 7, 14, 21 days).*

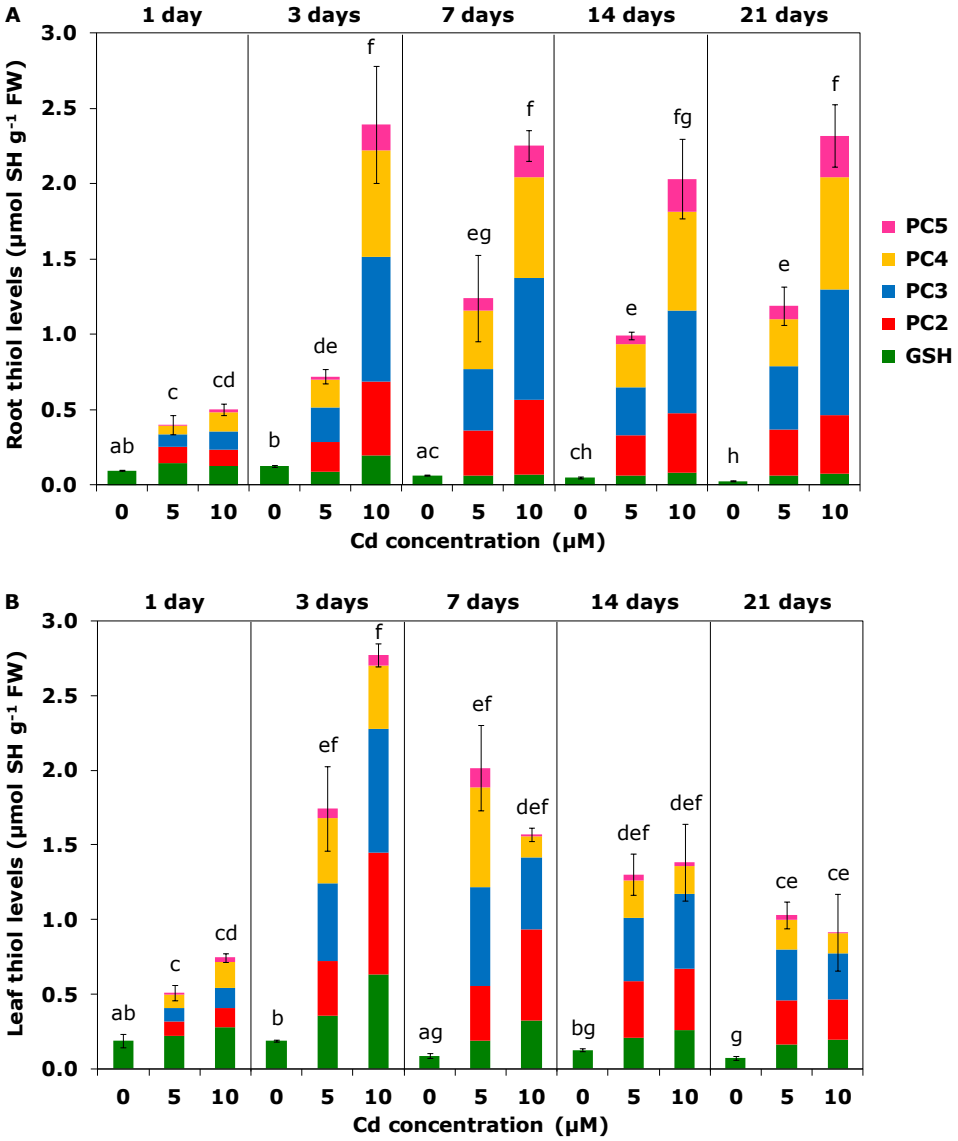


Fig. 7.5 Profiling the total glutathione [GSH ( )] and phytochelatins [PC2 ( ), PC3 ( ), PC4 ( ) and PC5 ( )] related reduced thiol content in Arabidopsis thaliana roots (A) and leaves (B) exposed to 0, 5 or 10 µM $CdSO_4$ in hydroponics for different time periods (1, 3, 7, 14, 21 days). Thiol content is expressed in GSH equivalents (nmol SH g^{-1} FW). Statistical significance in total thiol levels is expressed using lower case letters; n = 4 (two-way ANOVA, $P < 0.05$).

7.4.4 Glutathione deficiency limits vegetative growth after exposure to cadmium

Due to the major function of GSH in Cd chelation, GSH deficient mutants were included in this study to investigate the necessity of PCs in plant survival upon Cd exposure. Glutathione deficient *cad2-1*, *pad2-1* and *rax1-1* plants were exposed to Cd during two weeks in parallel with wild-type plants. Under control conditions, GSH deficiency did not result in phenotypical changes (Fig. 7.3, 7.7) or differences in fresh weight (Table 7.3), percentage dry weight (Fig. 7.8), root growth (Fig. 7.9) or rosette expansion (Fig. 7.10). Nevertheless, all GSH deficient mutants responded to a greater extent to Cd than the wild-type plants. First, exposure to Cd resulted in elevated leaf chlorosis and anthocyanin production in the mutants with respect to wild-type plants (Fig. 7.3 and 7.7). In addition, all mutants showed the formation of the inflorescence meristem after two weeks of exposure to 10 µM Cd (Fig. 7.7), while this process was only observed after three weeks in wild-type plants (Fig. 7.3). Secondly, dose-dependent decreases in fresh weight (roots and leaves) were significantly more profound in mutants than wild-type plants. Thirdly, the percentage dry weight after two weeks of Cd exposure increased dose-dependently in all genotypes (Fig. 7.8). However, in contrast to the mutants, the leaf dry weight percentage of wild-type plants was not increased after exposure to the lowest dose. Also, root growth analysis showed more than 50% growth reduction in mutant plants after exposure to 1 µM Cd in agar plates, while the roots of wild-type plants were only affected at higher Cd concentrations (Fig. 7.9). Finally, a reduced rosette expansion occurred dose-dependently in Cd-exposed wild-type plants, whereas the mutants showed rosette growth arrest upon exposure to both Cd concentrations (Fig. 7.10).

LEAVES (mg FW plant^{-1})			
genotype	**0 µM Cd**	**5 µM Cd**	**10 µM Cd**
wild-type	1 177 ± 95 ^{A}	378.1 ± 45.3 ^{B}	95.5 ± 9.2 ^{C}
cad2-1	1 250 ± 75 ^{A}	112.1 ± 14.3 ^{C}	97.7 ± 2.1 ^{C}
pad2-1	1 163 ± 76 ^{A}	41.4 ± 8.4 ^{D}	54.3 ± 1.6 ^{DE}
rax1-1	931 ± 79 ^{A}	111.2 ± 2.2 ^{C}	68.7 ± 4.0 ^{CE}
ROOTS (mg FW plant^{-1})			
genotype	**0 µM Cd**	**5 µM Cd**	**10 µM Cd**
wild-type	338.3 ± 59.9 ^{a}	81.2 ± 9.9 ^{b}	14.12 ± 2.05 ^{c}
cad2-1	353.5 ± 49.9 ^{a}	15.0 ± 4.8 ^{c}	10.63 ± 0.94 ^{c}
pad2-1	430.3 ± 59.5 ^{a}	11.6 ± 1.8 ^{c}	9.03 ± 0.33 ^{c}
rax1-1	303.0 ± 52.2 ^{a}	11.2 ± 2.3 ^{c}	7.92 ± 0.36 ^{c}

Table 7.3 *Fresh weight (mg FW plant^{-1}) of roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0, 5 or 10 µM $CdSO_4$ in hydroponics for two weeks. Statistical significance is expressed using lower (roots) and upper case letters (leaves); n = 4 (two-way ANOVA, P < 0.05).*

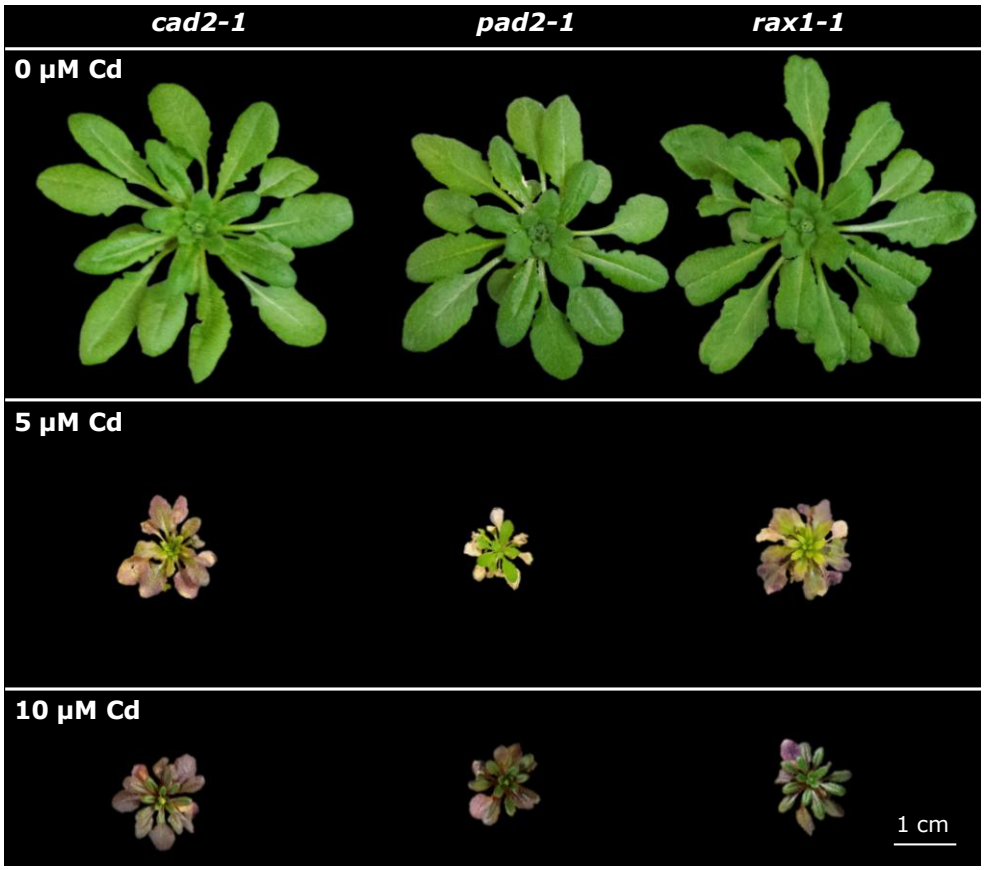


Fig. 7.7 *Representative pictures of the rosette appearance of Arabidopsis thaliana mutants (cad2-1, pad2-1 and rax1-1) exposed to 0, 5 or 10 µM $CdSO_4$ in hydroponics during two weeks; n = 14.*

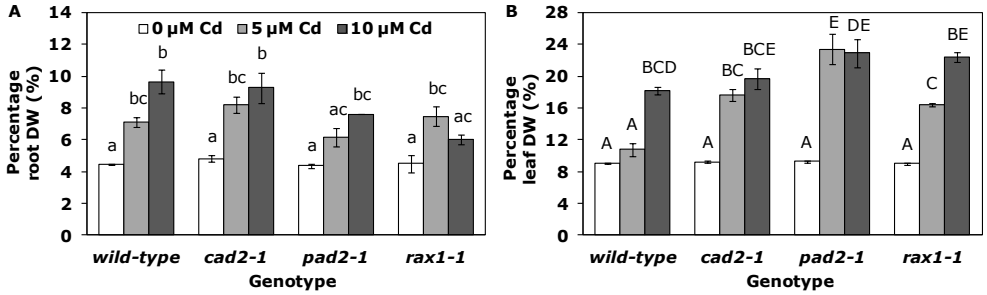


Fig. 7.8 *(A) Root and (B) leaf percentage dry weight (DW) of four Arabidopsis thaliana genotypes (wild-type, cad2-1, vtc1-1 and cad2-1 vtc1-1) exposed to 0 (□), 5 (▒) or 10 (■) µM $CdSO_4$ in hydroponics for two weeks. Statistical significance is expressed using lower (roots) and upper case letters (leaves); n = 4 (two-way ANOVA, P < 0.05).*

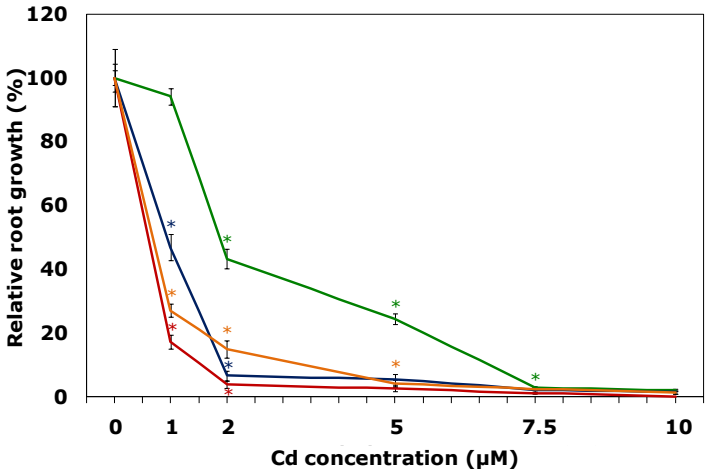


Fig. 7.9 *Root growth (expressed in% relative to 0 µM Cd) of four Arabidopsis thaliana genotypes [wild-type (■), cad2-1 (■), pad2-1 (■) and rax1-1 (■)] exposed to 0, 1, 2, 5 or 10 µM $CdSO_4$ for one week in agar plates. Statistically significant decreases with respect to a lower Cd concentration are indicated by * in the corresponding colour; n = 12 (Kruskal-Wallis, P < 0.05).*

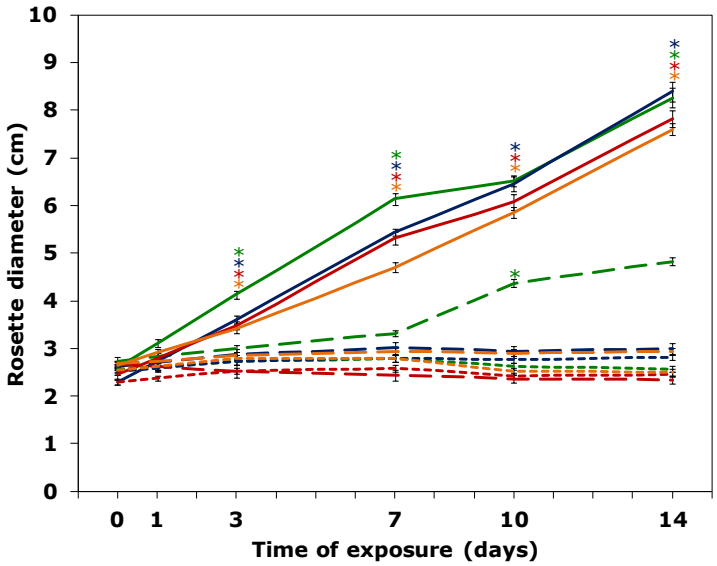


Fig. 7.10 *Rosette diameter of four Arabidopsis thaliana genotypes [wild-type (■), cad2-1 (■), pad2-1 (■) and rax1-1 (■)] exposed to 0 (——) or 5 (- - -) µM $CdSO_4$ in hydroponics for different time periods (0, 1, 3, 7, 10, 14 days). Statistically significant increases over time within each condition are indicated by * in the corresponding colour; n = 14 (one-way ANOVA, P < 0.05).*

7.4.5 Deficiency in glutathione influences the element profile in Arabidopsis after cadmium exposure

In GSH deficient mutants, element levels were only investigated at a fixed time point, *i.e.* after two weeks. Although no Cd levels were detected in control plants, all genotypes exposed to Cd possessed the same dose-dependent amount of Cd in their roots (Fig. 7.11A). The mutation in GSH1 however, negatively affected Cd translocation to the leaves, which decreased about 4.5- and 12.5-fold relative to wild-type plants exposed to 5 and 10 µM Cd, respectively (Fig. 7.11B). The mutant plants did not show significant differences in element concentrations under control conditions, compared to wild-type plants (Table 7.4). Although they responded in a similar way upon Cd exposure, mutant plants generally responded to a greater extent than wild-type plants. Root Cu levels increased in a dose-dependent manner in all genotypes, but *cad2-1* and *pad2-1* mutants showed significantly higher levels after 5 µM Cd and *rax1-1* mutants after 10 µM Cd exposure, in comparison to wild-type plants. In the leaves however, a dose-dependent decrease in Cu levels occurred to the same extent in all genotypes. Although leaf Fe levels did not show significant changes, root Fe levels were dose-dependently increased in all genotypes. Both *pad2-1* and *rax1-1* mutants showed significantly higher Fe levels than wild-type plants after exposure to 5 µM Cd. Concerning Zn levels, roots showed a similar response in all genotypes, *i.e.* a dose-dependent increase. In the leaves however, Zn levels were only decreased in wild-type plants. Calcium levels changed in a similar manner in all genotypes: increasing levels in the roots and decreasing levels in the leaves. Both Mg and K concentrations were decreased dose-dependently in the roots of all genotypes. Wild-type K levels however, were only significantly decreased after 10 µM Cd, while the mutants showed significantly lower K levels after exposure to both Cd concentrations. Concerning root Mg levels, all mutants showed significantly lower levels than wild-type plants, independent on the dose. In the leaves, K levels showed a decreasing trend, which was more pronounced in the mutants than wild-type plants. Leaf Mg levels were dose-dependently decreased in all genotypes, but *pad2-1* and *rax1-1* mutants showed significantly lower Mg levels after 5 µM Cd exposure, in comparison to wild-type plants.

Table 7.4 *Element concentrations (µg g^{-1} DW) of roots and leaves of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 0, 5 or 10 µM $CdSO_4$ in hydroponics for two weeks. Statistical significance is expressed using lower (roots) and upper (leaves) case letters; n = 4 (two-way ANOVA, P < 0.05).*

	ROOTS			Element	LEAVES			
genotype	0 µM Cd	5 µM Cd	10 µM Cd	(µg g^{-1} DW)	0 µM Cd	5 µM Cd	10 µM Cd	genotype
wild-type	8.35 ± 0.62 a	90.14 ± 4.40 b	176.76 ± 11.14 cd		6.43 ± 0.17 A	6.34 ± 0.21 ABC	2.52 ± 0.47 CDEF	***wild-type***
cad2-1	11.01 ± 0.87 a	144.47 ± 17.24 de	186.27 ± 9.95 cd	**Cu**	4.00 ± 1.17 BCE	3.61 ± 0.40 CDF	1.27 ± 0.09 F	***cad2-1***
pad2-1	9.65 ± 0.12 a	224.58 ± 21.21 cf	228.68 ± 13.32 cf		5.32 ± 0.24 AB	4.16 ± 0.92 ACD	1.78 ± 0.17 DEF	***pad2-1***
rax1-1	7.74 ± 0.54 a	112.92 ± 8.11 be	282.14 ± 20.00 f		4.97 ± 0.17 AC	3.01 ± 0.14 CDEF	1.29 ± 0.02 DF	***rax1-1***
wild-type	1 134 ± 193 a	2 793 ± 220 b	8 154 ± 796 c		78.40 ± 7.49 A	70.92 ± 39.05 A	120.51 ± 57.97 A	***wild-type***
cad2-1	947 ± 36 a	5 950 ± 1104 bc	7 817 ± 929 c	**Fe**	39.74 ± 13.60 A	74.12 ± 46.29 A	23.32 ± 11.12 A	***cad2-1***
pad2-1	997 ± 181 a	8 742 ± 928 c	8 990 ± 869 c		59.95 ± 13.69 A	97.43 ± 36.57 A	47.88 ± 14.36 A	***pad2-1***
rax1-1	926 ± 201 a	6 006 ± 737 c	11 185 ± 828 c		50.45 ± 6.27 A	23.24 ± 3.01 A	20.71 ± 5.84 A	***rax1-1***
wild-type	60.07 ± 5.83 a	244.61 ± 12.36 b	417.18 ± 13.39 cd		48.52 ± 4.61 AB	65.38 ± 2.73 B	21.15 ± 6.32 C	***wild-type***
cad2-1	71.87 ± 2.79 a	339.50 ± 7.78 bc	450.93 ± 45.13 cd	**Zn**	28.51 ± 8.25 AC	34.80 ± 2.88 AC	7.83 ± 0.60 C	***cad2-1***
pad2-1	62.34 ± 5.27 a	434.50 ± 12.20 cd	517.08 ± 28.28 d		31.94 ± 2.21 AC	38.90 ± 13.50 AC	11.17 ± 0.75 C	***pad2-1***
rax1-1	53.97 ± 2.74 a	267.69 ± 13.35 b	496.27 ± 44.68 d		28.78 ± 1.53 AC	31.18 ± 2.45 AC	7.58 ± 0.44 C	***rax1-1***
wild-type	1 350 ± 137 a	2 794 ± 93 b	2 918 ± 218 b		25 764 ± 1262 A	24 139 ± 424 A	7655 ± 3148 BD	***wild-type***
cad2-1	1 345 ± 142 a	3 298 ± 128 b	3 780 ± 579 b	**Ca**	15 361 ± 4506 AD	12 900 ± 1 701 AC	2657 ± 168 CE	***cad2-1***
pad2-1	1 218 ± 112 a	3 584 ± 194 b	3 284 ± 102 b		21 800 ± 782 AB	10 015 ± 3 898 ACD	4351 ± 735 CD	***pad2-1***
rax1-1	1 090 ± 34 a	3 045 ± 224 b	2 770 ± 404 b		21 279 ± 331 AB	9 775 ± 958 ABC	2491 ± 155 DE	***rax1-1***
wild-type	1 753 ± 54 a	1 308 ± 72 b	1 002 ± 46 c		3 995 ± 248 AB	4 290 ± 259 A	1525 ± 612 CD	***wild-type***
cad2-1	1 665 ± 69 a	623 ± 7 d	575 ± 17 d	**Mg**	2 892 ± 856 AC	2 611 ± 360 ACD	471 ± 30 D	***cad2-1***
pad2-1	1 722 ± 51 a	604 ± 32 d	573 ± 23 d		4 007 ± 176 AB	1 910 ± 884 BCD	731 ± 103 CD	***pad2-1***
rax1-1	1 922 ± 40 a	728 ± 60 cd	486 ± 89 d		4 525 ± 189 A	1 855 ± 180 BCD	437 ± 20 D	***rax1-1***
wild-type	41 043 ± 2044 a	31 512 ± 1780 ab	19 218 ± 774 bc		12 741 ± 504 AB	15 425 ± 494 A	7275 ± 1430 BC	***wild-type***
cad2-1	37 128 ± 2743 a	14 781 ± 7092 cde	7 166 ± 850 cd	**K**	9 398 ± 2733 ABC	11 205 ± 553 ABC	3937 ± 275 C	***cad2-1***
pad2-1	38 834 ± 1251 a	6 462 ± 460 cd	5 194 ± 999 d		13 142 ± 156 AB	10 757 ± 3 676 ABC	5735 ± 628 BC	***pad2-1***
rax1-1	44 180 ± 2089 a	21 574 ± 2573 be	5 048 ± 38 cd		14 833 ± 425 A	10 775 ± 680 ABC	4127 ± 112 C	***rax1-1***

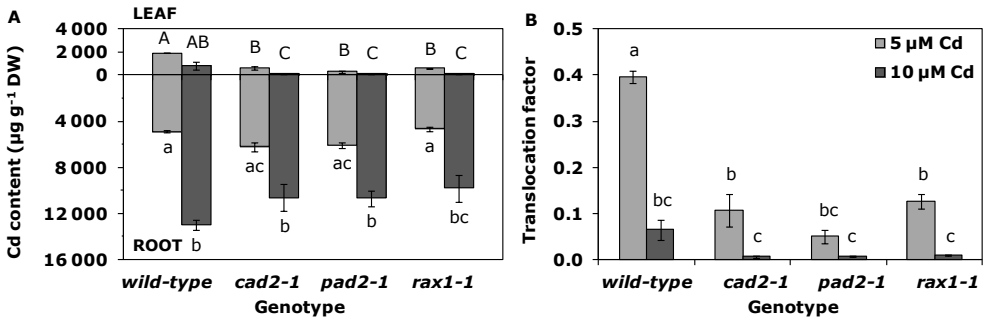


Fig. 7.11 *(A) Root and leaf Cd concentrations (µg g^{-1} DW) and (B) the corresponding translocation factor from roots to shoots of four Arabidopsis thaliana genotypes (wild-type, cad2-1, pad2-1 and rax1-1) exposed to 5 (□) or 10 (■) µM $CdSO_4$ in hydroponics for two weeks. Note the different scales on the y-axes between both organs. Statistical significance is expressed using lower (roots and translocation factor) and upper (leaves) case letters; n = 4 (two-way ANOVA, P < 0.05).*

7.5 Discussion

The development of refined and sensitive biochemical and molecular techniques shifted the biological interest in plant responses to Cd exposure from morphology and physiology at the whole plant level during chronic exposure, to cellular and molecular processes in acute experimental set-ups. The latter however, often does not show growth disturbances or toxicity symptoms (Cuypers *et al.* 2011). In order to integrate short-term plant responses to Cd exposure into a larger context, a prolonged exposure of 3-week-old plants during another three weeks was studied using a phenotypic analysis in combination with Cd detoxification and mineral nutrition. The growth stages used in this analysis were adapted from the soil- (Boyes *et al.* 2001) and hydroponics-based (Keunen *et al.* 2011b) phenotypic analysis platforms described before. Our experimental timing using plants between three and six weeks old starts at growth stage 3, related to rosette growth, and ends after growth stage 5, which includes emergence of inflorescence. This process of vegetative to reproductive transition is a major developmental event in plants. In order to maximise reproductive success, the timing of this switch is regulated by an interplay between developmental and environmental factors (He *et al.* 2010). In this study, control plants showed a strong vegetative growth at the level of biomass production (Table 7.3) and rosette expansion from the shoot

apical meristem (Fig. 7.2). After two weeks, the rosette growth reached a plateau and the meristem started to transform into an inflorescence meristem (Fig. 7.3), indicating plants' capacity for reproduction (Boyes *et al.* 2001).

7.5.1 Cadmium disturbs mineral nutrition and development of Arabidopsis thaliana plants

Arabidopsis thaliana wild-type plants were exposed to two environmentally realistic Cd concentrations (5 and 10 µM, Krznaric *et al.* 2009) in hydroponics (for a schematic overview see Fig. 7.12). Exposure to Cd resulted in multiple dose-dependent physiological disturbances. First, the observed decrease in fresh weight as a result of Cd accumulation (Table 7.1) was consistent with increasing dry weight percentages (Fig. 7.1). Together, this suggests a change in the plant's water status, *i.e.* decreased water uptake or elevated water loss, both of which may occur following Cd-induced membrane damage (Barcelo & Poschenrieder 1990). Another explanation for increased dry weight percentage is enhanced production of organic matter. It has been demonstrated before that exposure to Cd and other metals activates lignification processes in cell walls in order to increase Cd/metal-binding capacities of the cell wall and hence limit Cd/metal ions entering the cells (Chapter 5 and 6; Cuypers *et al.* 2002). Secondly, the uptake and translocation of mineral nutrients was significantly altered after exposure to Cd (Table 7.2), which has been described before (Liu *et al.* 2003; Nazar *et al.* 2012). For all investigated elements, significant changes in nutrient levels were observed when plants were treated with 10 µM Cd, whereas the effects were limited upon exposure to 5 µM Cd, except for elevated Cu levels in roots of Cd-exposed plants. In combination with decreasing Cu levels in the leaves, this points towards Cd-induced translocation problems, which might disturb CO_2 assimilation and ATP synthesis in the leaves (Yruela 2005). In general, the roots showed a more disturbed nutrient profile than the leaves. Notable, root K levels decreased strongly after three days of exposure to 10 µM Cd, which was less pronounced in the leaves. These decreasing K levels might indicate membrane destabilisation due to Cd-induced oxidative damage (Gussarsson & Jensen 1992; Demidchik *et al.* 2010), consistent with a possible disturbance in water status. All these factors have been associated with morphological changes including restricted plant growth or crop yield (Yadav 2010; Ghaffari *et al.* 2011). Root Cd accumulation increased with time and with

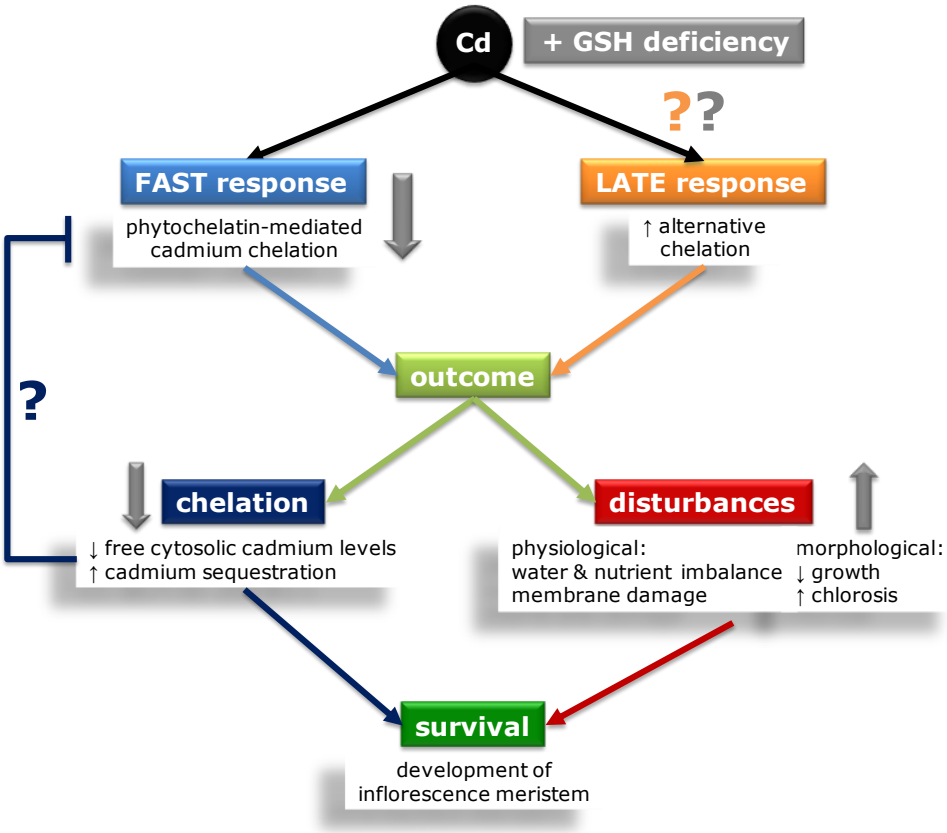


Fig. 7.12 *Schematic overview of Cd-induced responses over time in Arabidopsis thaliana. Grey arrows highlight the effect of GSH deficiency. The question marks indicate the proposed hypotheses in this study.*

their respective concentrations, whereas leaf Cd levels were constant and independent on the dose (Fig. 7.4). However, in both organs, visible time- and dose-dependent disturbances occurred at morphological level: retarded (5 µM Cd) or arrested (10 µM Cd) biomass production (Table 7.1) and rosette expansion (Fig. 7.2), and induction of chlorosis (Fig. 7.3). Regardless of these vegetative limitations, all plants entered growth stage 5 after three weeks (*i.e.* six weeks old), characterised by the transition into an inflorescence meristem. This confirms recent findings that *A. thaliana* plants exposed to 5 and 10 µM Cd in hydroponics were able to complete all stages of their life cycle, independent on the applied Cd concentration (Keunen *et al.* 2011b). This might be explained by the equal leaf Cd levels in all exposed plants (Fig. 7.4B), supporting the well-

known defence mechanisms in plants: Cd retention in roots and retranslocation from the leaves back to the roots (Van Belleghem *et al.* 2007; Nocito *et al.* 2011; Jozefczak *et al.* 2014).

7.5.2 Time-dependent decrease in phytochelatin-based cadmium chelation

Numerous studies in several non-accumulator plants like *A. thaliana* present convincing evidence for PC-mediated Cd tolerance. Both wild-type plants treated with the GSH1 inhibitor BSO as well as mutants deficient in GSH (*cad2-1*, *pad2-1*, *rax1-1*) or PCs (*cad1-3*) show increased sensitivity to Cd (Chapter 4, 5 and 6; Howden and Cobbett 1992; Schat *et al.* 2002; Zhang *et al.* 2013; Sobrino-Plata *et al.* 2014). The high affinity of Cd to the thiol groups on GSH and PC renders them major components in fast Cd chelation and sequestration, which is characterised by plant-internal GSH/PC to Cd molar ratios (SH/Cd) between 1:1 and 3:1 (Rauser 1990; de Knecht *et al.* 1995; Schat *et al.* 2002). In this study we were interested in the PC-mediated Cd tolerance in *A. thaliana* over time (summarised in Fig. 7.12). The fact that PCS is constitutively expressed and readily activated by GSH-Cd complexes (Vatamaniuk *et al.* 2000), enables fast and efficient PC responses after Cd exposure (Ebbs *et al.* 2002; Rauser 2003; Jozefczak *et al.* 2014) as observed during the first three days in this study (Fig. 7.5, 7.6). Only in roots exposed to the highest dose, Cd accumulation was larger than the chelating capacity by PCs. The resulting Cd-induced stress is reflected by the growth arrest in this condition (Table 7.1, Fig. 7.2).

Previous findings suggested a time-dependent decrease of leaf PC levels after prolonged Cd exposure (Chapter 3; de Knecht *et al.* 1995; Jozefczak *et al.* 2014). In order to assign this observation to either efficient Cd sequestration, which reduces the need for chelation, or to system failure and eventually lethality, a long-term study was performed. After three days, SH/Cd ratios decreased over time, reaching levels below 1:1 (Fig. 7.6). In leaves, this resulted from constant Cd levels over time and decreased PC levels, whereas roots showed constant PC levels despite increasing Cd contents (Fig. 7.4, 7.5). Since PC synthesis in roots responded to Cd in a dose-dependent manner, saturation of PCS activity is excluded. Activation of PCS requires post-translational activation by cytosolic Cd binding to the enzyme itself (Vatamaniuk *et al.* 2000). Therefore, a viable hypothesis is that decreases in free cytosolic Cd

concentrations over time directly affects PCS activity and hence PC levels. This coincides with the results observed in the leaves, while the constantly increasing root Cd levels suggest a steady-state between PCS activation and cytosolic Cd levels. This hypothesis might suggest PC-mediated Cd chelation with efficient Cd sequestration into metabolically less active sites. According to the model of Vögeli-Lange and Wagner, the latter is associated with PC degradation and recycling to the cytosol (Vögeli-Lange & Wagner 1990; Zenk 1996). Compartmentalisation of Cd into less sensitive tissues and organelles is a well-known detoxification mechanism (Choi *et al.* 2001; Clemens 2006; Isaure *et al.* 2006; Huguet *et al.* 2012). In addition, a decrease in free cytosolic Cd availability might result from an extra cytosolic-bound chelation mechanism other than PCs. Several studies have proposed alternative mechanisms for Cd chelation including metallothioneins or still unidentified non-protein thiols (S-ligands) and carboxyl or hydroxyl groups of organic acids (O-ligands) (de Knecht *et al.* 1995; Schat *et al.* 2002; Koren *et al.* 2013). Studies have even suggested a time-related shift in Cd-chelating compounds from S-ligands, including PCs in young tissues, to O-ligands in mature and senescent leaves (Küpper *et al.* 2004; Koren *et al.* 2013). Although this shift has only been described in Cd tolerant plants (Callahan *et al.* 2006), our findings suggest an analogous change of ligand dominance in Cd sensitive plants. This shift might partially be explained by the high energetic cost that is associated with a fast PC response (Steffens 1990). In conclusion, this study supports that PC-mediated Cd tolerance is a fast and efficient response in *A. thaliana* (Jozefczak *et al.* 2014). The decreasing SH/Cd ratios that followed after short-term exposure were not associated with system failure since all plants were able to survive and develop inflorescence meristems (Fig. 7.3). Instead, we hypothesise that both Cd sequestration into less sensitive sites and non-PC cytosolic-bound chelation mechanisms contribute to decreasing levels of free cytosolic Cd ions, which in its turn reduced the need for further increases in PC levels.

7.5.3 Glutathione deficiency increases plant's sensitivity to cadmium but does not prevent plant survival

In agreement with previous studies, our data concerning biomass production (Table 7.3), percentage dry weight (Fig. 7.8) and rosette development (Fig. 7.7, Fig. 7.10) demonstrated that the reduced GSH levels in *cad2-1*, *pad2-1* and

rax1-1 mutants did not affect the normal fitness of *A. thaliana* plants (Howden *et al.* 1995a; Cobbett *et al.* 1998; Parisy *et al.* 2007). The absence of phenotypic effects has been associated with the capacity to maintain high mitochondrial GSH levels in these mutants, allowing normal plant development (Zechmann *et al.* 2008). Exposure to Cd however, confirmed the generally accepted increased Cd sensitivity in GSH deficient mutants (Howden *et al.* 1995a; Cobbett *et al.* 1998; Koffler *et al.* 2014), which was reflected in decreased biomass production (Table 7.3), more severe signs of chlorosis and anthocyanin production (Fig. 7.7), rosette growth arrest (Fig. 7.10) and reduced root growth (Fig. 7.9) at lower Cd concentrations and enhanced nutritional disturbances (Fig. 7.4), in comparison to wild-type plants (for a schematic overview see Fig. 7.12). Interesting differences in leaf Cd levels were observed between wild-type and mutant plants: GSH deficiency, and hence a decreased capacity for PC synthesis, was associated with a reduced Cd translocation to the aerial parts of the plants (Fig. 7.11). This decrease has been observed before (Sobrino-Plata *et al.* 2014) and is consistent with the long-distance translocation of Cd from roots to shoots in a PC-dependent manner (Gong *et al.* 2003).

Nevertheless, despite the increased Cd sensitivity, all mutants developed an inflorescence meristem after two weeks of exposure to 10 µM Cd (Fig. 7.7). This indicates their capacity for reproduction and hence survival (Boyes *et al.* 2001). Clearly, Cd-exposed plants developed their inflorescence meristems earlier than control plants, which is in line with accelerated senescence that has been associated with abiotic stresses like Cd (Sandalio *et al.* 2001; Kolář & Seňková 2008; Keunen *et al.* 2011b).

7.5.4 Conclusion

This study supports the importance of GSH for the protection of plants against Cd stress. The absence of significant PC production in the mutants increased Cd-induced toxicity symptoms, but despite the vegetative growth disturbances, they were still able to survive the Cd-imposed stress. Together, the data support the hypothesis that after a fast PC response, plants activate alternative mechanisms that complement these early Cd detoxification processes in a chronic experimental set-up.

Chapter 8

General discussion

Main findings and new hypotheses formed throughout this research project are presented in the following general discussion.

8.1 Research framework

During the Industrial Revolution in the 18th century, the development of advanced industrial and agricultural technologies resulted in the current worldwide metal pollution (Järup & Akesson 2009). Due to the construction of three zinc (Zn)-smelters in the Campine region in Belgium, cadmium (Cd) is one of the metals that progressively accumulated in the environment (Scokart *et al.* 1983; Lauwerys *et al.* 1990). Despite its non-essential character, the chemical similarity between Cd and essential cations like Zn, calcium (Ca) and iron (Fe), results in non-specific Cd uptake and translocation in plants. This bioaccumulation of Cd is a serious threat for the food chain and hence the human population and the entire ecosystem (Leonard *et al.* 2004; Chary *et al.* 2008; Nair *et al.* 2013).

In plants, Cd has detrimental effects at the level of morphology (e.g. decreased crop yield) and physiology (e.g. disturbed photosynthesis) (Sanita di Toppi & Gabbrielli 1999; Clemens *et al.* 2002). At the cellular level, Cd elicits an oxidative challenge characterised by a disturbance in the cellular redox state, *i.e.* a dynamic balance between pro- and antioxidants. Elevated production of reactive oxygen species (ROS) upon Cd exposure has been associated with both its detrimental oxidising effects and its protective signalling function to increase the levels of antioxidants (Foyer & Noctor 2005a; Cuypers *et al.* 2012b). The antioxidant system consists of enzymes [e.g. superoxide dismutase (SOD), ascorbate peroxidase (APx), catalase (CAT), redoxins] and metabolites [e.g. glutathione (GSH), ascorbate (AsA)] (Hancock *et al.* 2001; Halliwell 2006). The current work was focused on two abundant, multifunctional metabolites, GSH and AsA. They are key players in plant defence responses upon Cd exposure via their functions as antioxidant and redox buffer (Bielen *et al.* 2013). In addition, GSH is a major component in Cd chelation due to the high affinity of Cd for its thiol (SH) group on cysteine, and as a precursor for phytochelatins (PCs) (Jozefczak *et al.* 2012). Highly reduced GSH and AsA pools are essential in order to perform these functions. Therefore, the oxidised form of GSH (*i.e.* glutathione disulfide, GSSG) and AsA (*i.e.* dehydroascorbate, DHA) are reduced by the high-capacity enzymes glutathione reductase (GR) and dehydroascorbate reductase (DHAR), respectively (Foyer & Noctor 2011).

Within our research group, a hydroponic cultivation method has been optimised and statistically validated to study environmental stress conditions in a controlled manner after short- (Smeets *et al.* 2008b) and long-term exposure (Keunen *et al.* 2011b) of *Arabidopsis thaliana* plants. The main objective of this study was to increase our knowledge on the functions of GSH and AsA in the model organism *A. thaliana* during sublethal Cd exposure. To this end, kinetic measurements were performed in wild-type *A. thaliana* plants to unravel the spatiotemporal involvement of GSH and AsA during Cd detoxification (Chapter 3), which provided a solid basis for more in-depth research in the following chapters. The specific role of both metabolites was further explored by comparing Cd-induced responses in GSH and/or AsA deficient mutants with wild-type plants after short (Chapter 4) and prolonged exposure times (Chapter 5). In order to investigate specific details on how GSH might regulate alternative antioxidant mechanisms during Cd stress, multiple GSH deficient mutants were exposed to Cd during both short and prolonged exposure times (Chapter 6). Finally, the importance of fast PC-mediated Cd chelation was examined during a long-term Cd exposure (Chapter 7). A working-model representing the observed Cd-induced responses in *A. thaliana* wild-type and mutant plants is shown in Fig. 8.1.

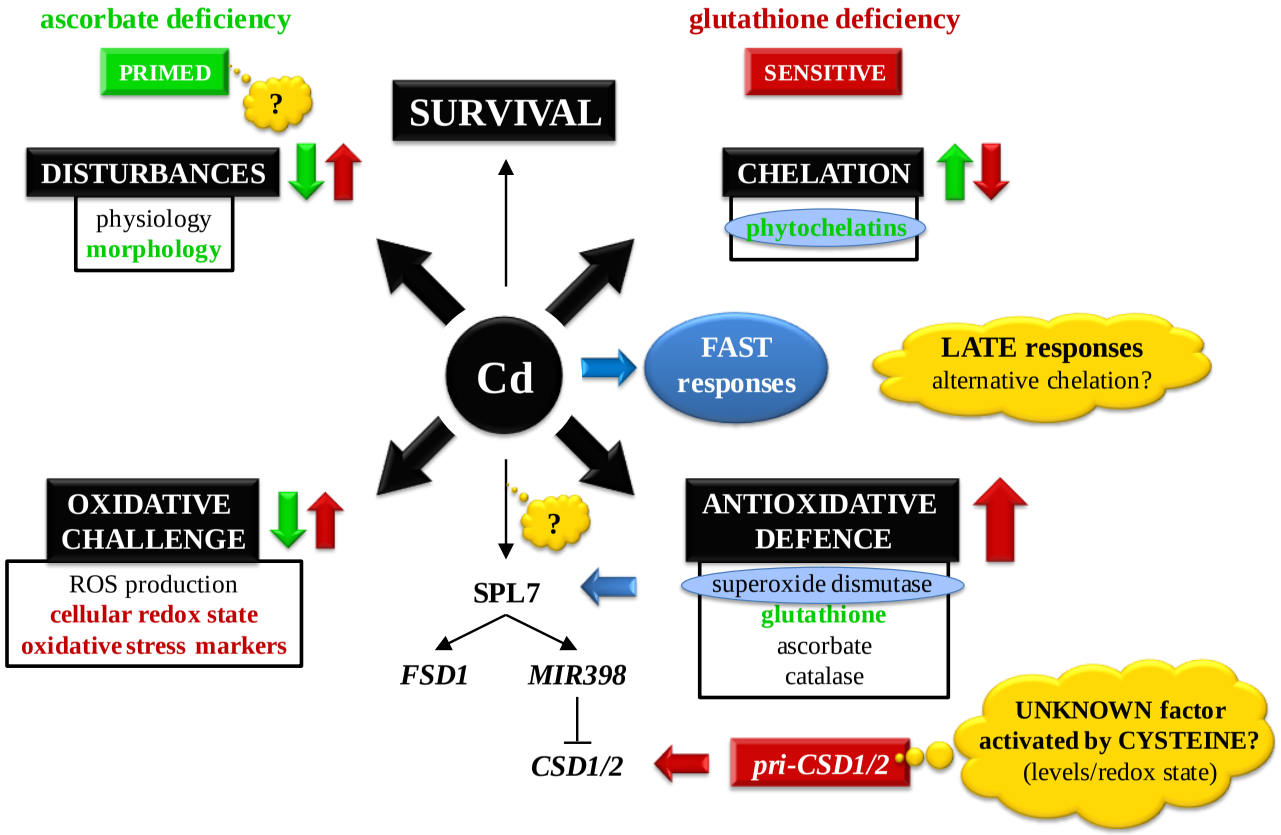


Fig. 8.1 *Schematic overview of the suggested working model of both genotype- and Cd-specific responses in Arabidopsis thaliana plants. Genotype-specific responses under control conditions are highlighted in green (ascorbate deficient plants) or red (glutathione deficient plants) font. Cadmium-induced responses that are more (arrow upwards) or less (arrow downwards) pronounced in the mutant with respect to the wild-type plants are indicated in green (ascorbate deficient plants) or red (glutathione deficient plants) arrows. Fast responses are highlighted with blue circles. Yellow clouds represent interesting topics for future studies.*

8.2 Spatiotemporal involvement of glutathione and ascorbate reveal differential responses of *Arabidopsis thaliana* leaves and roots to cadmium-detoxification

Cadmium-induced disturbances on plant physiology and morphology are well-documented (Skorzynska-Polit *et al.* 2010; Cuypers *et al.* 2012a). Although many targets have been identified, the spatiotemporal progression of the events occurring in Cd-exposed tissues remains largely unclear. Therefore, this study investigated short- (hours), prolonged (days) and long-term (weeks) Cd-induced defence mechanisms in *A. thaliana* roots and leaves. In line with previous studies, exposure to different environmental Cd concentrations (5 and 10 µM; Krznaric *et al.* 2009) was toxic to wild-type plants. This was reflected in a time- and dose-dependent manner at the level of growth parameters (Fig. 3.2, 7.2, 7.3, Table 7.1), nutrient balance (Table 7.2) and oxidative stress [lipid peroxidation (Fig. 3.3), oxidative cellular redox environment (Fig. 5.4, Table 6.2), hallmark genes for oxidative stress (Table 5.2)]. Nevertheless, all wild-type plants were able to develop their inflorescence meristem, indicating their capacity for reproduction and hence survival (Fig. 7.3).

Plants posses multiple Cd-induced detoxification mechanisms, of which chelation and antioxidative defences are two major systems that were studied in the present work. A kinetic screening in Chapter 3 upon acute Cd exposure (2, 24, 48, 72 h) revealed that both PC production and the regulation of SOD are primary responses in roots. A fast and strong accumulation of Cd in roots was observed in a time- and dose-dependent manner (Fig. 3.1), which continued during long-term exposure (Fig. 7.4). Once inside root cells, Cd is preferentially chelated with high-affinity ligands like the thiol group on the cysteine residue of GSH (Rauser 2003). In order to provide sufficient thiols in roots, GSH was immediately (*i.e.* after 2 h) consumed for PC production before *de novo* GSH synthesis was activated (Fig. 3.4). This high affinity of Cd to thiols is characterised by plant-internal GSH/PC to Cd molar ratios (SH/Cd) between 1:1 and 3:1 (Rauser 1990), as evidenced in wild-type plants exposed to Cd between one day and one week (Fig. 7.6). A fast and efficient PC response upon Cd exposure was possible by the fact that phytochelatin synthase (PCS) is constitutively expressed and readily activated by GSH-Cd complexes (Vatamaniuk *et al.* 2000). This direct and fast protection mechanism against free

Cd ions however, resulted in a transient GSH depletion (Fig. 3.5), possibly limiting the direct and indirect ROS scavenging capacity of GSH. In addition, alternative antioxidative pathways including CAT and AsA were found to be activated at a later time point to complement GSH's antioxidative functions and hence ensure efficient neutralisation of Cd-induced ROS (Table 3.1, Fig. 3.6, 3.7). In time, GSH biosynthesis was able to keep up with the high demand for thiols and GSH levels restored dose-dependently (Fig. 3.5). Together, these findings suggest that chelating and antioxidant capacity were biphasic in roots. Besides PCs, microRNA398 (MIR398)-directed SOD regulation was also activated at transcription level after 2 h of exposure in roots, resulting in SOD responses (Table 3.2, Fig. 3.6), preceding other antioxidant pathways (Table 4.2).

In Cd-exposed leaves however, generally delayed and minor responses were observed with respect to the roots. This might result from the immediate PC-mediated response in roots after 2 h, which limits Cd translocation to the leaves (Fig. 3.1, 7.4). Whereas GSH was found to be the key metabolite affected in roots of Cd-exposed plants, both GSH and AsA pathways were addressed in the leaves (Table 4.2, Fig. 5.3, 5.4). Consistent with leaf GSH and AsA levels that were higher than in roots (Fig. 3.5, 3.7), leaves were in a generally less stressed state (Table 5.2, Fig. 5.3, 5.4). Finally, the observed changes in the GSH redox state in roots (Fig. 3.5) might have generated a fast inter-organ redox signal that triggered the leaves to prepare for the impending stress (Foyer & Noctor 2005b; Nocito *et al.* 2006).

Regardless of increased toxicity and vegetative limitations in wild-type plants exposed to the highest dose, long-term experiments confirmed that plants were equipped with efficient Cd and ROS detoxification mechanisms in order to preserve plant survival (Fig. 7.3). The chronic (1, 3, 7, 14, 21 days) experimental set-up in Chapter 7 supported a fast PC-mediated Cd tolerance in *A. thaliana* (Fig. 7.5). However, this response was followed by decreased SH/Cd ratios (Fig. 7.6). It was hypothesised that the need for further stimulation of PC synthesis was reduced in time, by decreasing levels of free cytosolic Cd ions. The latter might result from both Cd compartmentalisation into less sensitive sites and an alternative chelation mechanism, other than PCs. Leaf trichomes have been reported to accumulate high amounts of Cd without damaging the cells (Isaure *et al.* 2006). Additionally, including GSH deficient mutants in the

long-term experiment revealed that despite their lack of significant PC production (Fig. 6.3) and hence increased Cd-induced toxicity symptoms (Table 7.3, 7.4, Fig. 7.8, 7.10), they were still able to survive (Fig. 7.7). Together, these results suggest that after a fast PC response, Cd-exposed plants activate alternative mechanisms that complement this early Cd detoxification process.

8.3 Ascorbate deficient mutants are continuously primed and hence prepared for additional cadmium stress

The fact that previous studies link biotic resistance in AsA deficient plants with constitutive priming (Conklin & Barth 2004; Pavet *et al.* 2005; Mukherjee *et al.* 2010), led to the hypothesis that these mutants are also more tolerant to abiotic stresses like Cd toxicity. In agreement with previous studies, *vtc1-1* mutants exhibited slow-growth phenotypes under control conditions (Table 4.1, 5.1, Fig. 4.1, 5.1), but no oxidised cellular redox state was observed (Fig. 5.3, 5.4), confirmed by similar transcript levels of oxidative stress marker genes as compared to wild-types (Fig. 5.6) (Veljovic-Jovanovic *et al.* 2001; Olmos *et al.* 2006). Our data present evidence for an increased antioxidant (Fig. 4.2, 4.5), signalling (Fig. 4.5) and chelating (Fig. 4.2, 5.5) capacity in *vtc1-1* plants under control conditions, which is suggested to render the mutants less sensitive to Cd-induced oxidative stress. It is hypothesised that elevated thiol (GSH and PC) levels under both control and exposed conditions, primarily protect *vtc1-1* plants against Cd stress. This was evidenced by unaltered growth (Fig. 4.1, 5.1) and fresh weight (Table 4.1, 5.1) and transcriptionally equal or less responsive profiles in *vtc1-1* mutants with respect to wild-type plants (Table 4.2, 5.2, Fig. 4.4, 5.7). In conclusion, the continuous priming caused the AsA deficient mutants to compensate their deficiency with elevated GSH and hence PC levels, which are key agents in fast chelating and subsequent antioxidative processes.

8.4 Glutathione deficient mutants perceive permanent oxidative stress and hence activate multiple alternative antioxidant mechanisms upon cadmium exposure

In agreement with previous studies, 60 to 80% reduction in GSH levels did not cause phenotypic changes (Parisy *et al.* 2007). However, GSH deficient mutants did suffer from oxidative stress under control conditions, as indicated by a more oxidised GSH pool (Fig. 5.4, Table 6.2) and inductions of oxidative stress hallmark genes (Fig. 5.6). In addition, GSH deficiency was not compensated with elevated basal defence capacities, explaining the increased Cd-sensitivity in GSH deficient mutants (Howden *et al.* 1995a; Cobbett *et al.* 1998). Both short- and long-term exposure revealed elevated Cd-induced toxicity in the mutants in comparison to wild-type plants at the level of growth (Fig. 5.1, 7.7, 7.10, Table 6.1, 7.3), nutrient balance (Table 7.4) and oxidative challenge [oxidative cellular redox environment (Fig. 5.4, Table 6.2), hallmark genes for oxidative stress (Table 5.2)]. Nevertheless, the GSH deficient mutants were able to survive the Cd-imposed stress (Fig. 7.7), suggesting a key role for GSH during short-term exposure, which is successfully complemented over time with alternative mechanisms.

The severe deficiency in both Cd chelation via PCs (Fig. 4.7, 5.5, 6.3) and ROS neutralisation via GSH (Fig. 4.2, 5.4, Table 6.2) resulted in accelerated and strong activations of alternative antioxidant pathways in Cd-exposed GSH deficient mutants, with respect to wild-type plants. The alternative mechanisms observed in this work involve SOD, CAT, the AsA-GSH cycle and other GSH-related pathways (Fig. 4.2, 4.8, 6.4, 6.5, Table 4.2, 5.2, 6.3, 6.4). These findings support the fact that a decreased cellular GSH redox state [*i.e.* decreased GSH levels and GSH/GSSG ratio (Fig. 4.2, 5.4, Table 6.2)] contributes to the regulation of antioxidant gene expression (Apel & Hirt 2004; Mittler *et al.* 2004).

Chapters 3, 4 and 5 point towards both PCs and the transcriptional regulation of SOD as early responses in wild-type plants under Cd stress. In this context, it is not surprising that deficiency in one of these mechanisms (e.g. GSH deficiency) strongly triggers the activation of the other mechanism (*i.e.* SOD). One FeSOD (*FSD1*) and two CuZnSOD (*CSD*) genes have shown to be regulated by the squamosa promoter-binding protein-like 7 (SPL7) transcription factor upon Cd

exposure (Cuypers *et al.* 2011; Gayomba *et al.* 2013). This transcription factor stimulates the expression of *FSD1* and *MIR398b/c*, while the latter is known to target *CSD1/2* transcripts (Nagae *et al.* 2008; Yamasaki *et al.* 2009). All investigated genotypes, showed SOD responses at transcriptional level that are consistent with SPL7 activation (Table 3.1, 4.2, 5.2, 6.2). However, Chapter 4 revealed that *cad2-1* mutants exposed to 5 µM Cd during 24 h strongly stimulated their *CSD1/2* expression, resulting in an increase in total SOD activity despite the up-regulation of *MIR398b/c* that was similar to wild-type plants (Fig. 4.8, Table 4.2). However, this transcriptional response was not observed after 72 h (Table 5.2). For this reason, a more in-depth experimental set-up involved multiple GSH deficient mutants (*cad2-1*, *pad2-1* and *rax1-1*) and both 24 and 72 h of 5 µM Cd exposure. In agreement with results obtained in the *cad2-1* mutant (Chapter 4), *CSD1/2* transcripts were significantly increased in the roots of all GSH deficient mutants after 24 h of Cd exposure (Fig. 6.5), which resulted in significantly increased SOD activities after 24 and/or 72 h (Fig. 6.4). Additionally, expression of primary transcripts of *CSD1/2* (*pri-CSD1/2*) was included as a measure for transcription rate. Strikingly, *pri-CSD1/2* were strongly up-regulated in roots after 24 h, to a significantly greater extent than in wild-type plants. Consistent with the findings in the *cad2-1* mutant (Chapter 5), this was a fast but transient effect that attenuated over time (Table 5.2). This SOD stimulation, overruling *CSD* repression by *MIR398*, in Cd-exposed plants under GSH deficient conditions has not been reported before. This discovery offers new insights and opportunities to unravel the role of GSH in regulating antioxidative defences during stress conditions. On the one hand, GSH deficiency coincided with a more oxidised cellular environment (Fig. 5.4, Table 6.2) and a well known accumulation of cysteine (Parisy *et al.* 2007). On the other hand, Cd exposure causes a cellular redistribution of GSH (Kolb *et al.* 2010). Together, these facts suggest that the (subcellular) thiol redox state (*i.e.* both concentration and reduced-oxidised ratio) contributes to the transcription rate of *CSD1/2* upon Cd exposure, pointing towards a yet unknown factor regulating *CSD1/2* expression.

8.5 Future outlook

In general, our data highlighted the essential role of GSH in plant's response to Cd exposure. However, the priming that was observed in AsA deficient mutants also deserves further investigation. Although elevated GSH levels have been observed before in AsA deficient mutants (Veljovic-Jovanovic *et al.* 2001), the underlying mechanism remains unknown. Chapters 4 and 5 exclude a transcriptional activation of GSH or PC biosynthesis genes under both control (Table A.4.3) and Cd-exposed conditions (Table 4.2, 5.2). However, investigating the activity of the corresponding enzymes could serve as a starting point for future research.

Long-term exposure to Cd suggested the activation of alternative mechanisms, which complement GSH and PCs in fast chelation and sequestration of Cd ions. Therefore, the question was raised which compounds are involved in this alternative Cd chelation (Fig. 7.12). According to the literature, metallothioneins (MTs) or other still unidentified non-protein thiols (S-ligands) are potent Cd chelators (de Knecht *et al.* 1995; Cobbett & Goldsbrough 2002). In Chapter 3, a dose-dependent up-regulation primarily of *MT2a* was demonstrated in both organs after 24 h of Cd exposure, which continued until 72 h (Table A.3.3). However, to further unravel the importance of MTs during short- and long-term Cd exposure, MTs should be determined at transcriptional and activity level over time. In addition, carboxyl and hydroxyl groups of organic acids and cell wall components (O-ligands) should be included in future studies due to their potential relevance in Cd chelation in older tissues (Küpper *et al.* 2004; Koren *et al.* 2013). Moreover, specific tissue, cellular and subcellular localisation of these S- and O-ligands would increase the current knowledge on Cd chelation.

Glutathione deficient mutants seemed to perceive permanent oxidative stress and hence were more sensitive to Cd. On the one hand, these mutants were still able to survive this Cd toxicity (Fig. 7.7). Therefore, it would be interesting to investigate the influence of low GSH levels on plant's reproductive capacity. To this end, the long-term experiment of Chapter 7 should be expanded until seed maturation in order to investigate the influence of low GSH levels on the production of germinative seeds under control and Cd conditions.

On the other hand, GSH deficiency activated alternative antioxidant mechanisms upon Cd exposure, in comparison to wild-type plants (Fig. 4.9, 5.9). Apart from GSH and PCs, the MIR398-directed SOD pathway was also found to be activated during early responses to Cd-induced ROS production. Our findings confirm a recent study suggesting that Cd triggers SPL7-dependent responses in *A. thaliana* (Gayomba *et al.* 2013). Although different mechanisms are speculated, future research should focus on how exposure to Cd induces SPL7 activity. In Chapter 6, we focused on the differential *CSD* regulation between wild-type and GSH deficient plants (Fig. 6.4, 6.5). The (subcellular) thiol redox state was suggested to be involved in the activation of an unknown transcriptional activator of *CSD1/2*, which overruled *CSD* repression by *MIR398* in Cd-exposed GSH deficient plants. In order to test this hypothesis, the subcellular localisation, quantification and redistribution of both reduced and oxidised forms of GSH and cysteine should be investigated in control and Cd conditions in both wild-type and GSH deficient plants. An interesting technique is the use of location-specific reduction-oxidation sensitive green fluorescent protein (roGFP) probes, which allows monitoring of dynamic redox events, in real time, *in vivo* (Jiang *et al.* 2006; Schwarzländer *et al.* 2009; Schnaubelt *et al.* 2014). Additionally, promoter studies (e.g. yeast-one-hybrid, electrophoretic mobility shift assay) would provide more information on the binding site and/or identification of the unknown factor that was suggested to transcriptionally activate *CSD1/2* expression.

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Scientific contributions

This section summarises scientific contributions that were made during the course of this PhD

International journals

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Acknowledgments

Dankwoord

In this section, I would like to guide you through my personal PhD experiences.
This creates a good opportunity to thank the people
that supported me along the way.

First, I would like to take the opportunity to thank my co-authors and the members of my PhD Jury for both their comments and practical contributions to my work.

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Mijn doctoraat speelde zich ook af buiten de muren van onze universiteit, zo kreeg ik verschillende mogelijkheden om naast congressen, cursussen en workshops, ook technieken te leren in het buitenland. In mijn eentje heb ik op deze manier Madrid en Amsterdam verkend en, samen met Eline, Parijs ontdekt. Mijn eerste keer op uitstap als IWT'er blijft **hét** topmoment van mijn doctoraatsstudie. Dit was in de zomer van 2010 waarin ik voor twee weken naar de "Universidad Autónoma de Madrid" in Cantoblanco mocht gaan om *in-gel* activiteiten te leren bij Professor Luis Hernández. Mijn eerste vlucht alleen duurde gelukkig niet lang, bovendien was ik gerust want Professor Hernández zou me opwachten aan de luchthaven. En inderdaad, "Professor" werd al snel "Luis" en hij zorgde ervoor dat ik me meteen thuis voelde in hun labo. Op mijn eerste werkdag leerde ik de Argentijnse Laura en de Spanjaard Juan kennen. Juan was volgens mij "de Tony" van het labo, altijd even vriendelijk en bereid zijn kennis te delen. Laura heeft me niet enkel technieken geleerd in hun labo, ze heeft mij ook onder haar vleugels genomen. Ze leerde mij de gewoontes van daar en gidste me doorheen de stad. Mijn ochtendritueel bestond uit een lekkere cappuccino van Starbucks tijdens mijn ochtendwandeling en metrorit naar de campus, zalig!! Niet te vergeten, Spanje werd wereldkampioen terwijl ik daar was. Al interesseert voetbal mij niet, de halve finale en de finale heb ik gesupporterd en genoten van de bruisende stad vol uitgelaten Spanjaarden. Luis kwam ik achteraf regelmatig tegen in Diepenbeek of op congressen, elke keer geraakten we aan de praat en dan kwamen al die mooie herinneringen weer naar boven. Dankjewel Luis, Laura en Juan!

Mi primer viaje como doctorando del IWT continúa siendo la parte más memorable de esta etapa. Esto tuvo lugar en el verano de 2010, cuando viajé por mi cuenta a la Universidad Autónoma de Madrid en Cantoblanco para aprender sobre actividades "in-gel" de la mano del profesor Luis Hernández. Por suerte, mi primer vuelo en solitario no duró mucho tiempo y estaba tranquila porque el profesor Luis Hernández me recogería en el aeropuerto. Al poco tiempo, el tratamiento de "profesor" dejó en su lugar un simple "Luis", a la par que éste me hacía sentir como en casa en su laboratorio. En mi primer día de trabajo, conocí a la Laura la argentina y a Juan, español. Siempre he pensado que Juan fue como "El Tony" del laboratorio; siempre amable y dispuesto a compartir sus conocimientos. Laura, no sólo me enseñó nuevas técnicas en el

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