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SUMMARY

Antropogenic activities can have a negative impact on the natural environment. Mainly industrial or agricultural areas have to cope with elevated levels of metals in the soil, air, (ground-) water and sediments. Although the overall response of plants to heavy metal exposure is quite similar and results in growth reduction and disruption of physiological processes such as photosynthesis and respiration, it is of great importance to expand our knowledge regarding the cellular and molecular processes influenced by metal toxicity.

The main objectives of the present work were: (1) the investigation of transcriptional and enzymatic alterations related to oxidative stress during heavy metal toxicity and (2) the study of their underlying signalling cascades. For this purpose, (sub)cellular effects of metals with distinct characteristics, e.g. essential versus non-essential, redox-active versus not redox-active were studied during short-time exposure.

In order to allow simple exposure to water-soluble toxic substances and to be able to study both roots and shoots simultaneously, the first experimental part of this work evaluates the optimalisation and statistical validation of the hydroponic set-up (**chapter 3**). To compare different treatments, we suggest a hydroponic culture system where factors that influence variabililty such as the usage of different pots and harvesting on different times of the day are taken into account in the analyses. Our results further highlighted the importance of a continuous aeration of the nutrient solution.

To meet the aimed objectives, ROS-producing and antioxidative components were analysed and compared after Cd and Cu treatment at both transcriptional and enzymatic level (**chapter 4, 5 and 6**). Plant responses differed between roots and shoots depending on the stress intensity. In roots, metal exposure resulted in high stress intensity presumably sensed by the plasma membranes leading to strong increases of lipid peroxidation. Cadmium strongly stimulated a NADPH-oxidase-dependent superoxide ($O_2^{\circ-}$) and hydrogen peroxide (H_2O_2) burst. Copper, on the other hand, did not influence NADPH-oxidase expression, nor was a Cu-induced H_2O_2 response detected. In the leaves, the metal stress intensity was milder because of the lower metal concentrations. Plasma

membranes, mitochondria and chloroplasts are suggested as potential sensing sites and an important role for lipoxygenases and NADPH oxidases was suggested in both stress situations.

Inside the cell, ROS are balanced against antioxidative defence mechanisms and ROS signalling is controlled by production and scavenging. Regarding the antioxidative defence system, metal-specific patterns of SOD-transcription were detected in both roots and shoots, where FSD1 expression was strongly upregulated in the roots of Cd-exposed plants, accompanied by a miRNA398-regulated CSD downregulation. The gene expression level of most of the H_2O_2-quenching enzymes was equally induced by both metals.

Secondary messengers such as ROS also induce the transcription of signal transduction mechanisms, which was the case under both stress conditions. In the roots, exposure to Cu resulted in an immediate activation of the signalling pathway at the transcriptional level. A more delayed (only at the highest exposure concentration) and less intense MAPKinase induction was observed in roots of Cd-exposed plants (**chapter 7, 8 and 9**). During Cd-stress, a strong simultaneous upregulation of NADPH oxidases and regulatory genes (*ANP1, ANP2, WRKY25,* …) suggested that the cooperation of these enzymes is essential in the activation of an appropriate defence response. Copper strongly induced both antioxidative genes *(GST2, CAT1, …)* as well as regulatory genes *(OXI1, MPK3, CDPK1 …)* at all exposure concentrations.

In the leaves, the stress-signalling mechanisms differed from the roots. Cadmium exposure strongly increased several MAPK components at the transcriptional level, whereas an upregulation of only one MAPK (*MEKK1*) was observed in the leaves of the Cu-exposed plants. Interestingly, *OXI1* (MAPK) and *WRKY25* (transcription factor) showed similar expression patterns both in roots of Cd-exposed plants and in leaves of Cu-exposed plants. Because Cu can enhance the production of $^{\circ}OH$ (Fenton reaction) directly in the roots, and $^{\circ}OH$ content was also elevated in the leaves of Cd-exposed plants, this radical presumably is common in the regulation of *WRKY25* and *OXI1* expression.

As this OXI1 is known as a key link between oxidative burst signals (such as H_2O_2) and downstream responses, we further unravelled its specific role in Cd and Cu stress. In **chapter 10**, a highly important role for OXI1 in the cellular

redox signalling during Cu-induced toxicity in the roots of *Arabidopsis thaliana* was described. Cadmium-exposed *oxi1* mutants were able to induce other signalling cascades, such as Ca^{2+}-dependent proteins, but no major role for OXI1 was hypothesized.

In conclusion, the data obtained demonstrate that even under environmentally realistic metal concentrations, the signal sensing and hence the cellular response is strongly dependent on the stress intensity and the chemical properties of the metal applied. Clear differences in cellular responses were also observed between roots and shoots. Specific metal-related responses can be used as potential biomarkers, especially the processes related to signal transduction such as the metal specific miRNA398 up/down regulation.

SAMENVATTING

Zware metalen zoals Cd en Cu komen van nature voor in de verschillende compartimenten van ons leefmilieu. Door antropogene activiteiten waaronder de metaalverwerkende industrie en de landbouw zijn de gehalten aan deze elementen vooral in bodem en water drastisch toegenomen en kunnen ze een belangrijke bedreiging vormen voor zowel planten, dieren als de mens. De fysiologische effecten van Cd en Cu op planten zijn reeds uitvoerig onderzocht en omvatten een verstoring van processen zoals fotosynthese, respiratie en transpiratie. Als voornaamste onderliggende cellulaire mechanismen hiervan werden het vervangen van essentiële cofactoren, het binden van beide metalen aan carboxyl- en sulfhydrylgroepen en de inductie van oxidatieve stress beschreven. Oxidatieve stress is een verstoring van de (cellulaire) redoxbalans in het voordeel van de pro-oxidanten. Een verhoogd gehalte aan reactieve zuurstofvormen leidt enerzijds tot schade, maar kan anderzijds ook specifieke (sub)cellulaire processen activeren in functie van herstel.
Het doel van dit werk betreft het ontrafelen van de onderliggende cellulaire en moleculaire mechanismen na blootstelling aan zware metalen met bijzondere aandacht voor de specifieke rol van reactieve zuurstofvormen hierin. Om dit te bestuderen werden de responsen in *A. thaliana* na blootstelling aan twee metalen met verschillende redoxreactieve eigenschappen, met name Cd (een niet-essentieel element) en Cu (en essentieel element), met elkaar vergeleken.

In een eerste deel werden de effecten van Cd en Cu op zowel ROS-producerende als antioxidatieve parameters bepaald (**hoofdstukken 4, 5 en 6**). Beide metalen vertoonden onderling een groot verschil in toxiciteit, waarbij na blootstelling aan gelijkaardige concentraties Cu meer toxisch bleek voor de plant dan Cd. Verder werden voor beide metalen duidelijke verschillen in stressresponsen vastgesteld tussen wortel en blad.
De wortels, *i.e.* de plaats van de blootstelling, vertoonden blijkbaar een hoger niveau van stress, wat gesuggereerd werd door o.a. een sterke stijging van de lipidenperoxidatie. Koper kan, omwille van zijn redoxreactieve eigenschappen, rechtstreeks reactieve zuurstofvormen induceren via de Fenton reactie. Cadmium daarentegen, is niet redoxreactief en veroorzaakt oxidatieve stress via

indirecte mechanismen, zoals bijvoorbeeld via een significante stijging in NADPH oxidase transcripten, of via een inhibitie van de antioxidatieve verdediging (CuZn superoxide dismutase). Desalniettemin leek de plasmamembraan in beide stress-situaties een belangrijke sensor, daar de genexpressie van lipoxygenase1 zeer sterk werd geïnduceerd door zowel Cd als Cu.
In het blad kwamen zowel de plasmamembraan, de mitochondria als de chloroplasten als potentiële sensoren van stress naar voor. Plasmamembraangebonden enzymcomplexen zoals NADPH oxidasen werden geactiveerd hetzij door beide metalen, hetzij door metaalgeïnduceerde signaalmoleculen. Ook hier leken lipoxygenasen een belangrijke rol te spelen.
Om hun redoxbalans terug in evenwicht te brengen activeren planten hun antioxidatief verdedigingssysteem. De waargenomen verschillen in de 'oxidatieve stress inductie' resulteerden in de activatie van specifieke antioxidatieve componenten. De CuZnSOD-transcriptie, bijvoorbeeld, werd geïnhibeerd door Cd maar sterk opgereguleerd door Cu. De FeSOD-expressie daarentegen werd dan weer sterk geïnduceerd door Cd in de wortels. Bovendien werden een aantal van deze verschillen gehandhaafd wanneer de planten werden blootgesteld in een multipollutie context (aan Cd en Cu gelijktijdig).

Secundaire signaalmoleculen zoals ROS zijn in staat signaaltransductiecascades te activeren. Mogelijks ligt de inductie van specifieke ROS-patronen aan de basis van verschillen in regulatie die op hun beurt resulteren in de metaalspecifieke activatie van het antioxidatief verdedigingssysteem (**hoofdstukken 7, 8 en 9**). Onze resultaten wijzen op een belangrijke rol van zowel het MAPK- als het Ca^{2+}-afhankelijke signaaltransductienetwerk in beide stress situaties. Desalniettemin werden ook hier metaal- en orgaanspecifieke nuances waargenomen. Naast deze signalisatiecascades, spelen ook andere regulatiemechanismen een belangrijke rol in de activatie van de antioxidatieve verdediging. In deze studie werden metaalspecieke effecten geobserveerd in de expressie van miRNA398, dat ondermeer de CuZnSOD-expressie reguleert.
Het laatste luik (**hoofdstuk 10**) beschrijft een detailstudie uitgevoerd naar de specifieke rol van OXI1, een redoxgevoelig MAPkinase, tijdens Cd en Cu stress. OXI1 bleek een belangrijke rol te spelen als signaalmolecule in wortels van planten blootgesteld aan Cu. Weinig verschillen werden waargenomen in de

OXI1-gemuteerde Cd-behandelde *A. thaliana* zaailingen, dewelke waarschijnlijk beter in staat zijn andere signaalmechanismen te activeren (zoals Ca^{2+}-afhankelijke kinasen).

Samenvattend kan worden geconcludeerd dat zelfs een korte termijn blootstelling aan lage metaalconcentraties duidelijke patronen van stressresponsen induceert. Significante verschillen tussen beide metalen werden aangetoond in deze studie, met als voornaamste verschil de miRNA398- en overeenkomstige CuZnSOD-expressie. Deze discrepanties zouden uiteindelijk kunnen leiden tot het vinden van specifieke biomerkers. Ze zouden dan ook de focus kunnen vormen voor verder intensief wetenschappelijk onderzoek in functie van specifieke biomerkers.

Table of Contents

List of abbreviations

ACT	actine
ACS	1-aminocyclopropane-1-carboxylate synthase
ADH	alcohol dehydrogenase
AGO	argonaute
ANP	NPK1-related protein kinase
AOX1A	alternative oxidase
APx	ascorbate peroxidase
ARE	antioxidant responsive element
AsA	ascorbate
Ca	calcium
CaM	calmoduline
CAT	catalase
Cd	cadmium
CDPK	calcium-dependent protein kinase
CSD	copper zinc superoxide dismutase
Cu	copper
DEPMPO	5-diethoxyphosphoryl-5-methyl-1-oyrroline N-oxide
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DTT	dithiothreitol
DW	dry weight
EPR	electron paramagnetic resonance
Fe	iron
FSD	iron superoxide dismutase
FW	fresh weight
GAPDH/GAPC	glyceraldehyde-3-phosphate dehydrogenase
GPx	glutathione peroxidase/guaiacol peroxidase
GR	glutathione reductase
GSH	glutathione
GSH1	γ-glutamylcysteine synthetase
GSH2	glutathione synthetase 2
GSSG	glutathione disulphide
GST	glutathione-S-transferase
GSTU	glutathione-S-transferase
Hg	mercury
H_2O_2	hydrogen peroxide
HSF	heat shock transcription factor
K	kalium
LDH	lactate dehydrogenase
LOX	lipoxygenase
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MEKK	MAPkinase kinase kinase
Mg	magnesium
MKK	MAPkinase kinase
Mn	manganese
MPK	MAPkinase
MRP	multidrug resistance-associated protein
MSD	manganese superoxide dismutase
MYB	transcription factor
Na	natrium
Ni	nickel
NIG1	NaCl-inducible gene 1
O_2	oxygen

$O_2^{\bullet-}$	superoxide
$^{\bullet}OH$	hydroxyl radical
$^1O_2$	singlet oxygen
OXI1	oxidative signal-inducible 1
P	phosphor
PA	phosphatidic acid
PAL	phenylalanine ammonia-lyase
PCS1	phytochelatin synthetase 1
PDC	pyruvate decarboxylase
PDK	phosphoinositide-dependent protein kinase
PLC	phospholipase C
PLD	phospholipase D
Px	peroxidase
RBOH	respiratory burst oxidase homolog
ROS	reactive oxygen species
S	sulphate
SOD	superoxide dismutase
SOS	salt overly sensitive protein (CBL-interacting protein kinase)
SPx	syringaldazine peroxidase
TBA	thiobarbituric acid
TBArm	thiobarbituric acid reactive metabolites
TCA	trichloric acetic acid
WRKY	DNA-binding protein (transcription factor)
ZAT12	H_2O_2-responsive zinc finger protein
Zn	zinc

Chapter 1

INTRODUCTION

1.1 General information on cadmium and copper

1.1.1 Chemical and physical properties

Cadmium is a soft, malleable, ductile, bluish-white bivalent metal (figure 1.1A), with the atomic number 48 and an atomic mass of 112.41 (table 1.1). The most common oxidation state is Cd(II), although rare examples of Cd(I) can be found. Cadmium has an ionic radius of 0.97 Å, which makes it almost identical to Ca^{2+} (with an ionic radius of 0.99 Å). Due to its chemical and physical properties (table 1.1), Cd is also closely related to Zn, and to a lesser extent to Hg. Cadmium is permanent in dry air, becomes coated with the oxide in moist air, burns on heating to redness, and is readily soluble in mineral acids. It is not redox active.

A. B.

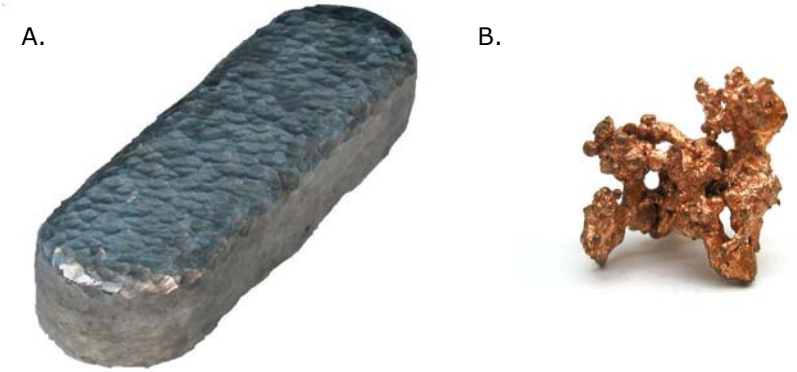
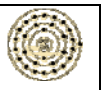


Figure 1.1: *Photographs of purified metallic cadmium (A) and native copper (B)*

Copper is a malleable, ductile, red metal (figure 1.1b). It has atomic number 29 and an atomic mass of 63.55 (table 1.1). Copper is a redox-active metal and exists in more than one oxidation state, among which the less stable Cu(I) state; and the more stable Cu(II) state. Under unusual conditions, a Cu(III) state and even an extremely rare Cu(IV) state can also be obtained. Because of its chemical and physical properties (table 1.1), Cu is one of the oldest metals

used in the industry. Copper is a metal that does not react with water, but the oxygen will react slowly at room temperature to form a layer of copper oxide on copper metal. It does react with hydrogen sulfide and sulfide containing solutions, and with a combination of oxygen and hydrochloric acid to form a series of copper chlorides.

Table 1.1: *Chemical and physical properties of Cd and Cu*

Properties	**Cadmium** 	**Copper** 
Chemical symbol	Cd	Cu
Atomic number	48	29
Atomic weight	112.41	63.546
Boiling point	765 °C	2567 °C
Conductivity, electrical	0.138 10^6/cm Ω	0.596 10^6/cm Ω
Conductivity, thermal	0.968 W/cmK	4.01 W/cmK
Density	8.64 g/cm³	8.96 g/cm³
Ionic radius	0.97 Å	0.73 Å
Melting point	321.18 °C	1084.6 °C
Series; group; period	Transition metal; 12; 5	Transition metal; 11; 4

1.1.2 Natural occurrence and biological role

Cadmium is a relatively rare element, and its abundance in the lithosphere is estimated at about 0.1-0.5 parts per million in the earth's crust (table 1.2). Its most common mineral greenockite (cadmium sulphide) is almost always associated with zinc sulphide (sphalerite). Nearly all major Zn deposits contain Cd in various amounts, with the ores containing a maximum of about 1%. A biological role for Cd has been discovered only recently, as a Cd-dependent carbonic anhydrase was found in marine diatoms (Lane *et al.* 2005, Park *et al.* 2007). Here, Cd exerts the same role as Zn in other anhydrases, but the diatoms live in environments with very low Zn concentrations, and their metabolism uses Cd rather than Zn.

Table 1.2: *Natural abundance of Cd and Cu*

Location	Cadmium abundance (ppm)	Copper abundance (ppm)
Earth's crust	0.1-0.5	50
Atlantic surface	0.0000011	0.00008
Atlantic deep	0.000038	0.00012
Pacific surface	0.0000011	0.00008
Pacific deep	0.0001	0.00025
Sun (Relative to $H=1E^{12}$)	71	1.15

Copper is a metallic element which is found in many locations. Copper occurs in native form (i.e. as nuggets of the free metal), in oxide ores (e.g. cuprite, Cu_2O), and sulphide ores (e.g. chalcocite (CuS_2); as chalcopyrite ($CuFeS_2$ or $Cu_2S.Fe_2S_3$) and bornite (Cu_3FeS_3). Copper is one of the most prevalent biological transition metals and plays a diverse role in all organisms, from bacteria to humans. Copper is found in a variety of enzymes, including the Cu centers of cytochrome c oxidase and the enzyme superoxide dismutase (containing Cu and Zn). In addition to its enzymatic roles, Cu is used for biological electron transport.

1.1.3 Industrial occurrence and production of cadmium and copper

Due to antropogenic activities many regions suffer from heavy metal pollution. Mainly areas with high industrial or agricultural activities have to cope with elevated levels of metals in the soil, air, (ground-) water and sediments.

Cadmium was first discovered in Germany in 1817 as by-product of the Zn refining process. Industrial applications for Cd were developed in the late 19th and early 20th century. Because Cd-containing products are rarely recycled, Cd emissions increased dramatically (Jarup 2003). Cadmium-sulfide based pigments were used as early as 1850 and also appeared prominently in the paintings of Vincent Van Gogh in the late 19^{th} century. Thomas A. Edison in the United States and Waldemar Junger in Sweden developed the first Ni-Cd batteries in the beginnings of the 20th century. Cadmium compounds are also used as corrosion-protection, as coating on steel and as stabilizers in PVC products. In agriculture, the use of artifical fertilizers highly contributes to soil and surface water contamination of Cd.

According to the British Geological survey, Chile was the top mine producer of Cu during the last years (with at least one-third world share), followed by the USA, Indonesia and Peru. Most Cu is mined as Cu sulfides extracted from ores from large open pit mines. Copper is essential in the electrical industry (Cu wire, electromagnets, electrical machines, …), it is used as an agricultural poison and as an algaecide in water purification. Furthermore, Cu is used in architecture (against corrosion), in household products (Cu plumbing, cookware and flatware), in musical instruments, as a component of coins, in analytical chemistry (Fehling's solution) and in medical research (e.g. radiation therapy).
In Belgium, severe Cd soil contamination was described. Historical emissions from the non-ferrous metal industry have contaminated the Meuse valley near Liège and the northern part of the Kempen with Cd due to zinc refining industry (Lauwerys *et al.* 1990, Bernard *et al.* 1992, Staessen *et al.* 1995). Nowadays, Cd is precipitated out of a electrolysis solution, which led to a strong decrease in Cd emission.

1.2 Toxicological effects in humans

Humans normally absorb Cd either by ingestion or inhalation. A recent study showed house dust as an important route of exposure to heavy metals in areas with contaminated soils (Hogervorst *et al.* 2006). Acute exposure to high Cd concentrations results in abdominal pain, vomiting, muscle cramps, vertigo, shock and loss of consciousness after oral exposure. Accidental acute inhalation of Cd causes chemical pneumonitis or metal fume fever. Chronic exposure to Cd causes pulmonary emphysema, renal complications (tubular damage, glomerular damage and kidney stones), bone disorders (osteomalacia and osteoporosis) and deregulated blood pressure (figure 1.2) (Jarup *et al.* 1998).
The Cadmibel study has shown that chronic environmental exposure to Cd in the north-east of Belgium was associated with about 30% increased urinary Cd excretion, osteoporosis, increased calciuria, renal dysfunction, and a 35% population-attributable risk of fractures. Cadmium is also classified as carcinogenic (group I) by the IARC (International Agency for Research on Cancer). Recently, the association between environmental Cd exposure and lung cancer was demonstrated (Nawrot *et al.* 2006).

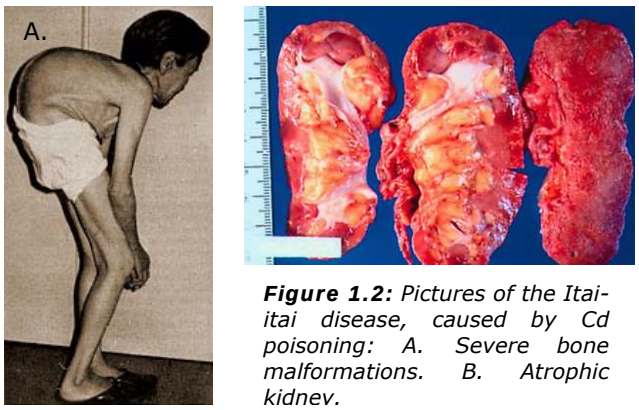


Figure 1.2: *Pictures of the Itai-itai disease, caused by Cd poisoning: A. Severe bone malformations. B. Atrophic kidnev.*

Copper is an essential element but increased Cu exposure can produce adverse health effects. Excess Cu can be found in many kinds of food, drinking water and air. Copper concentration in air is usually quite low, so that exposure to Cu through inhalation is negligible, except for the people that live near Cu smelters. Acute poisoning from ingestion of excessive Cu can cause gastrointestinal distress with symptoms such as nausea, vomiting and abdominal pain. Long term exposure to Cu causes irritation of the nose, mouth and eyes, but also headaches, stomachaches, dizziness, vomiting and diarrhea. High uptakes of Cu can result in liver and kidney damage and even death.

1.3 Cadmium and copper responses in plants

1.3.1 Uptake and transport

Uptake of metals in plants depends on their concentration and bioavailability in the soil, modulated by the presence of organic matter, pH, redox potential, temperature and the concentrations of other elements (Sanita di Toppi and Gabbrielli 1999, Kirkham 2006, Lock *et al.* 2007). Tight control of metal accumulation is essential and plants posses highly effective metal acquisition and uptake systems at both organismal and cellular level (Clemens *et al.* 2002). Uptake of metal ions is likely to take place through secondary transporters such as channel proteins and/or H^+-coupled carrier proteins. The membrane potential is negative on the inside of the plasma membrane and therefore provides a strong driving force for the uptake of cations through secondary transporters. Several cation transporters have been identified in the last years, most of them

with a rather broad substrate range. As such, Cd enters the cell through transport systems of other essential elements, e.g. Fe^{2+}, Ca^{2+} and Zn^{2+} (Clemens 2006). Copper, on the other hand, is an essential element and has high affinity for transport proteins among which transporters of the CTR family (Puig *et al.* 2007).

A striking feature found in the plant kingdom is the naturally selected hypertolerance, which can lead to a hyperaccumulation of metals to sometimes extraordinary quantities (Baker and Brooks 1989, Roosens *et al.* 2003). Established Cd hyperaccumulators are *T. caelurens* and *A. halleri* (Assunçao *et al.* 2003, Clemens 2006). A predominant Cu homeostasis function has also been attributed to the metal hyperaccumulator *T. caerulens* (Roosens *et al.* 2004).

1.3.2 Morphological and physiological effects

A fast inhibition of growth as well as decreases in photosynthetic activity, transpiration and respiration are common stress responses of plants exposed to heavy metals (Lagriffoul *et al.* 1998, Maksymiec *et al.* 2007). Overall, the phytotoxic effect of Cu on metabolism and growth of higher plants is mostly stronger as compared to other metals (Fargasova 2001, Wojcik and Tukiendorf 2003).

Cadmium is a non-essential element for plants that negatively affects growth and development. It interacts with the water balance (Perfus-Barbeoch *et al.* 2002, Vitoria *et al.* 2003) and influences stomatal opening, either via fewer stomata due to a slower cell replication in the plane of the epidermis or via a reduction of the number of open stomata (Zhu *et al.* 2005). Cadmium also damages the photosynthetic apparatus, in particular the light harvesting complex II, and the photosystem II and I (Krupa *et al.* 1993, Siedlecka and Krupa 1996, Sanita di Toppi and Gabbrielli 1999).

Copper is for plants an essential redox-active micronutrient that is involved in many physiological processes in plants and hence is required for normal growth and development (Yruela 2005). The redox properties that make Cu an essential element, also contribute to its inherent toxicity. Excess Cu can disturb normal development by adversely affecting physiological processes in plants (Maksymiec 1997). Decreased biomass production, especially in the roots, has

commonly been observed in plants subjected to Cu stress (Fargasova 2001, Drazkiewicz *et al.* 2004, Martins and Mourato 2006).

1.3.3 Cellular effects

1.3.3.1 Metal storage

To cope with excess quantities of metals plants have developed several mechanisms to maintain the cellular metal homeostasis. Main components are transport, chelation and sequestration processes (Clemens 2001).

In the cytoplasm, proteins such as phyochelatins, metallothioneins, ferritins and organic acids are responsible for the metal-ion complexation and detoxification (Cobbett 2000, Cazalé and Clemens 2001). Metallothioneins are small proteins and are identified in multiple organisms. Proposed functions of these ligands include detoxification of metals (especially Cu), cytosolic Zn buffering, and involvement in metal secretion via leaf trichomes (Clemens 2001). Metallothionein production is strongly influenced by metal ions among which Cd and Cu. Phytochelatins are a second important group of metal ligands, and also their synthesis is influenced by heavy metal ions (Cobbett 2000). Complexation with phytochelatins followed by compartmentalization in the vacuole, or possible precipitation in the cytoplasm, is suggested as the most important chelation pathway of Cd ions (Sanita di Toppi and Gabbrielli 1999, Cobbett 2000, Van Belleghem *et al.* 2007).

Removal of excess metals can be achieved by efflux or compartmentalization (after metal-ligand binding as described above) (Colangelo and Guerinot 2006). The main storage compartment for toxic compounds in plant cells is the vacuole (Clemens 2001, Bovet *et al.* 2003). Recent investigations also demonstrated a role for plasma-localized ABC transporters as efflux mechanisms for Cd ions (Klein *et al.* 2006, Kim *et al.* 2007). Metal efflux and transport is reviewed by Colangelo and Guerinot (2006).

1.3.3.2 Cellular mechanisms of metal ion toxicity

At the cellular level, Cd interaction with biomolecules such as proteins and nucleic acids, is known to affect enzyme activities and causes alterations in membrane permeability (Stohs and Bagchi 1995, Sanita di Toppi and Gabbrielli 1999). Disruption of cell integrity by Cd may explain the disproportionate loss of

cell K^+ often observed during Cd exposure. Furthermore, Cd inhibits the oxidative mitochondrial phosphorylation (Kesseler and Brand 1994, Kesseler and Brand 1995), negatively disrupts electron transport chains and causes a reduction in the chlorophyll biosynthesis. Possible underlying mechanisms are the binding of Cd to sulfhydryl and/or carboxyl groups (hence the importance of phytochelatins in the cellular defence against Cd) or the replacement of essential cofactors, e.g. Zn or Ca by Cd (*cfr*. 1.4) (Skorzynska-Polit *et al.* 1998, Perfus-Barbeoch *et al.* 2002).

Copper is a common cofactor for enzymes including oxidases and oxygenases. Copper acts as a catalyst in the formation of ROS (reactive oxygen species) and catalyzes production of membrane lipids causing destabilization of membranes and increase of their permeability. After a longer exposure to toxic Cu concentrations, chlorophyll concentration decreases which is associated with the simultaneous destruction of the inner structure of the chloroplast (Maksymiec 1997). Copper ions may also act indirectly on some enzymatic pathways of plant cells.

Both Cd and Cu are described as genotoxic. Metal-binding to cell nucleus or metal-mediated ROS production cause DNA base modifications, inter and intramolecular crosslinking of DNA and proteins, mutations and DNA strand breaks. Although oxidative damage explains most of the mechanisms involved in metal-mediated carcinogenity and acute toxicity, other pathways, such as DNA methylation and competition with other metals also should be considered.

1.4 Oxidative stress

At the cellular level, Cd and Cu induce oxidative stress (Cuypers *et al.* 2000, Smeets *et al.* 2005, Martins and Mourato 2006). Oxidative stress is a disturbance of the cellular redox status that is often observed in stress situations. ROS are also produced under normal circumstances and plant cells posses a well equipped antioxidative defence system to maintain the redox equilibrium (Mittler *et al.* 2004, Bhattacharjee 2005, Foyer and Noctor 2005a). Copper is a redox-active metal and is therefore able to produce ROS directly via Fenton and Haber-Weiss reactions (Schutzendubel and Polle 2002). Since Cd itself is not redox-active, the increased ROS levels are likely to be induced via

indirect mechanisms such as interaction with the antioxidative defence system, disruption of electron transport chains or induction of lipid peroxidation.

1.4.1 ROS production

Oxidative stress is mostly used as a negative term implying a harmful process, but ROS also regulate many physiological processes in plants (Mittler *et al.* 2004). Under normal circumstances (in non-stress conditions), ROS are generated as by-products of some biochemical processes but also have important cellular functions such as signal transduction. They are involved in the regulation of several processes, such as mitosis, tropisms and cell death (Foyer and Noctor 2005a, Kangasjärvi *et al.* 2005).

1.4.1.1 Activation of oxygen

Activation of oxygen may occur by two different mechanisms: via absorption of sufficient energy or via monovalent reduction (figure 1.3) (Edreva 2005, Halliwell 2006).

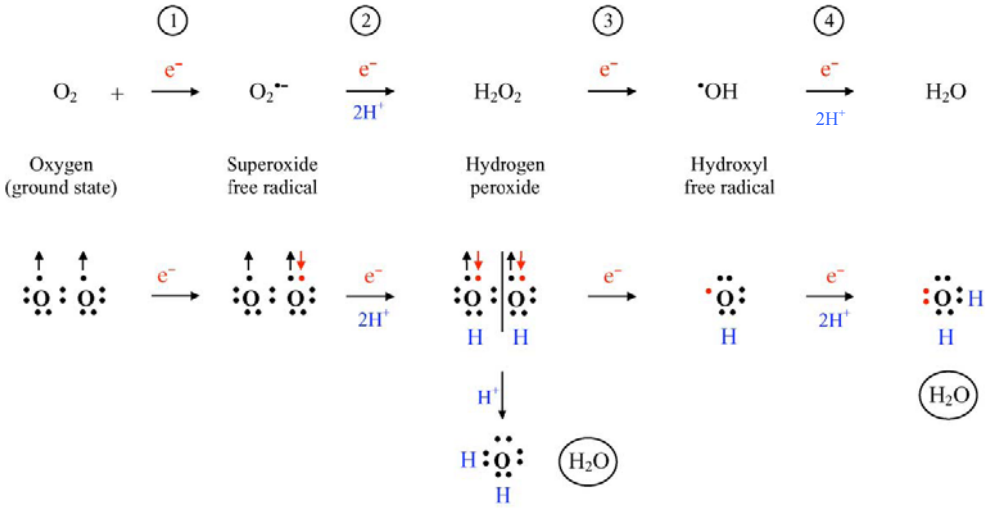


***Figure 1.3**: Consecutive four-step monovalent reduction of dioxygen yielding reactive oxygen intermediates and $2H_2O$ (source: Edreva, 2005)*

An input of energy overcomes the spin restriction to form the singlet state ($^1O_2$), which can directly oxidize proteins, DNA and lipids. The second mechanism of activation is by the stepwise monovalent reduction of oxygen (figure 1.3). If a single electron is supplied to O_2, superoxide ($O_2^{\bullet-}$) is formed. Addition of another

electron to $O_2^{\circ-}$ gives rise to O_2^{2-} and in biology the two-electron reduction product of O_2 is hydrogen peroxide (H_2O_2). H_2O_2 is not a free radical because all its electrons are paired, and both H_2O_2 and $O_2^{\circ-}$ are less damaging by themselves. They can, however, form the highly reactive hydroxyl radical ($^{\circ}OH$) via the Fenton/Haber Weiss reaction (Arora *et al.* 2002) (figure 1.4). Hydroxyl radicals are the strongest oxidizing agents known and can initiate lipid peroxidation, attack DNA, proteins and many other small molecules.

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\circ}$ (Fenton)

$O_2^{\circ-} + H_2O_2 \xrightarrow[Fe(III)/Cu(II)]{} O_2 + OH^- + OH^{\circ}$ (Haber Weiss)

Figure 1.4*: Fenton and Haber Weiss reaction*

The oxidation of organic substances may proceed by two possible reactions: via the addition of OH to the organic molecule or via the abstraction of a hydrogen atom from it. The hydrogen abstraction is best demonstrated by lipid peroxidation in cell membranes (figure 1.5) (Arora *et al.* 2002).

Initiation step:

$RH + OH^{\circ} \longrightarrow R^{\circ} + H_2O$
(Lipid alkyl radical)

Propagation step:

$R^{\circ} + O_2 \longrightarrow ROO^{\circ}$
(Lipid peroxy radical)

$ROO^{\circ} + RH \longrightarrow ROOH + R^{\circ}$

$ROOH \longrightarrow RO^{\circ} \longrightarrow$ Epoxides, hydroperoxides, glycol, aldehydes

Termination step:

$R^{\circ} + R^{\circ} \longrightarrow R + R$
(Fatty acid dimer)

$R^{\circ} + ROO^{\circ} \longrightarrow ROOR$
(Peroxide bridge dimer)

$ROO^{\circ} + ROO^{\circ} \longrightarrow ROOR + O_2$

Figure 1.5*: The chain reactions of lipid peroxidation.*

1.4.1.2 Places of ROS production

ROS arise in plant cells via a number of routes (figure 1.6). In photosynthetic plants, excess energy can lead to formation of a chlorophyll triplet state that can transfer its excitation energy onto O_2 to produce $^1O_2$ (Foyer *et al.* 1994, Asada 2006). Oxygen is also activated at other sites in the chloroplast, and production of reduced and exciting species of ROS in chloroplasts is reviewed by Asada (2006).

The mitochondrial electron transport chain is another major source of ROS production (Chen *et al.* 2003, Rhoads *et al.* 2006) and mitochondria are key players in plant cell redox homeostasis and signalling (Noctor *et al.* 2007). Redox sensing and signalling associated with both chloroplasts and mitochondria are integrated networks that are highly important in the regulation of cellular processes in both control and stress conditions (Sweetlove *et al.* 2002, Foyer and Noctor 2003, Navrot *et al.* 2007).

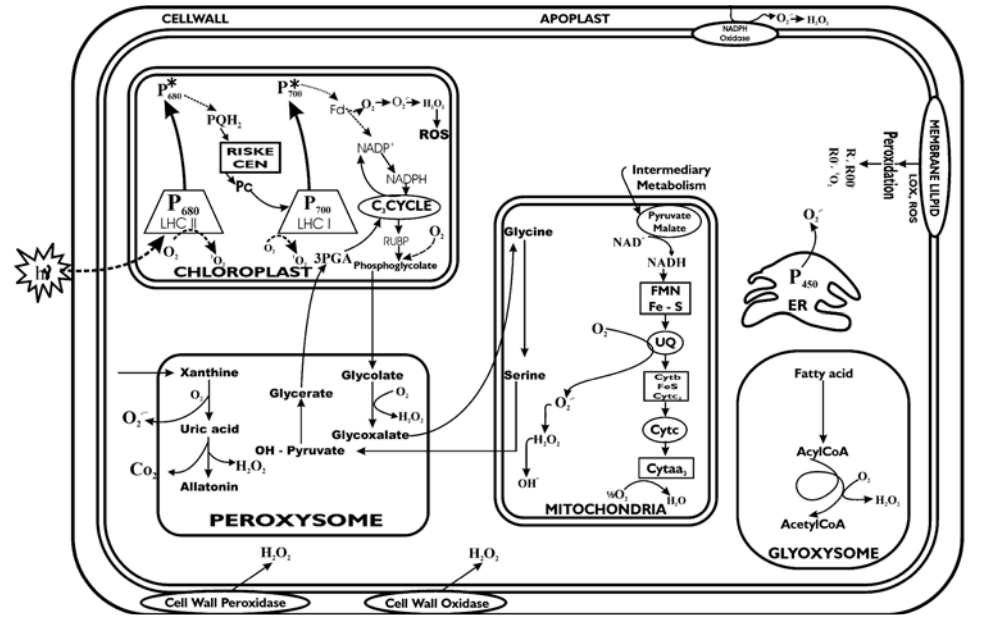


***Figure 1.6**: Sources of ROS in plant cells (source: Bhattacharjee, 2005)*

Peroxisomes also produce ROS, among which H_2O_2, $O_2^{\circ-}$ and NO°. Peroxisomal ROS are sometimes generated as a by-product, but can also function as signalling molecules and contribute to a more integrated communication among

cell compartments and tissues (del Rio *et al.* 2002, Foyer and Noctor 2003, del Rio *et al.* 2006).

In addition, ROS are produced by several oxidases present in the membrane and cytoplasm. Plant cell NADPH oxidases, for example, have analogue functions as the animal enzyme (Sagi and Fluhr 2006). They catalyze the production of $O_2^{\circ-}$ and are involved in multiple cellular processes. In addition, NADPH oxidases play a highly important role in the cellular responses against both abiotic and biotic stresses, among which Cd and Cu (Kwak *et al.* 2003, Torres and Dangl 2005, Torres *et al.* 2006, Yeh *et al.* 2007). As such, they can function as intercellular responders to create local ROS transients, possibly via the generation of a secondary messenger H_2O_2. In pea leaves, NADPH oxidase activity was shown to be the main source of H_2O_2 generation (via $O_2^{\circ-}$) after Cd exposure (Romero-Puertas *et al.* 2004, Maksymiec and Krupa 2005, Torres and Dangl 2005).

Lipoxygenases (LOX) catalyze the dioxygenation of polyunsaturated fatty acids producing hydroperoxy fatty acids which can be subsequently metabolized via several secondary pathways to produce bioactive compounds such as jasmonate and oxylipins (Porta and Rocha-Sosa 2002). LOXes are involved in physiological processes such as growth and development, and are often induced during specific stress conditions (Ali *et al.* 2005b). According to previous research, the increase in LOX activity could be important in the oxidative stress induction after Cd and Cu application (Somashekaraiah *et al.* 1992, Skorzynska-Polit and Krupa 2006, Skorzynska-Polit *et al.* 2006).

1.4.2 ROS scavenging

The steady state level of ROS in the different cellular compartments is determined by the interplay between multiple ROS-producing pathways and ROS-scavenging mechanisms. ROS and other oxidants are balanced against the antioxidative defence system (figure 1.7) which is composed of enzymes as well as metabolites as ascorbate (AsA), glutathione (GSH) and α-tocopherol. Sequential and contemporaneous action of these antioxidative metabolites with catalases (CAT), superoxide dismutases (SOD) and peroxidases (PXs) makes up the cells antioxidative power, that maintains the cellular redox homeostasis within certain limits (Mittler *et al.* 2004). All subcellular compartments contain specific antioxidative enzymes or metabolites which are coordinated and

controlled by underlying regulatory mechanisms. The balance between SOD, and APX (ascorbate PX) (and/or CAT) activity in cells is considered to be crucial for determining the steady state level of $O_2^{\bullet-}$ and H_2O_2.

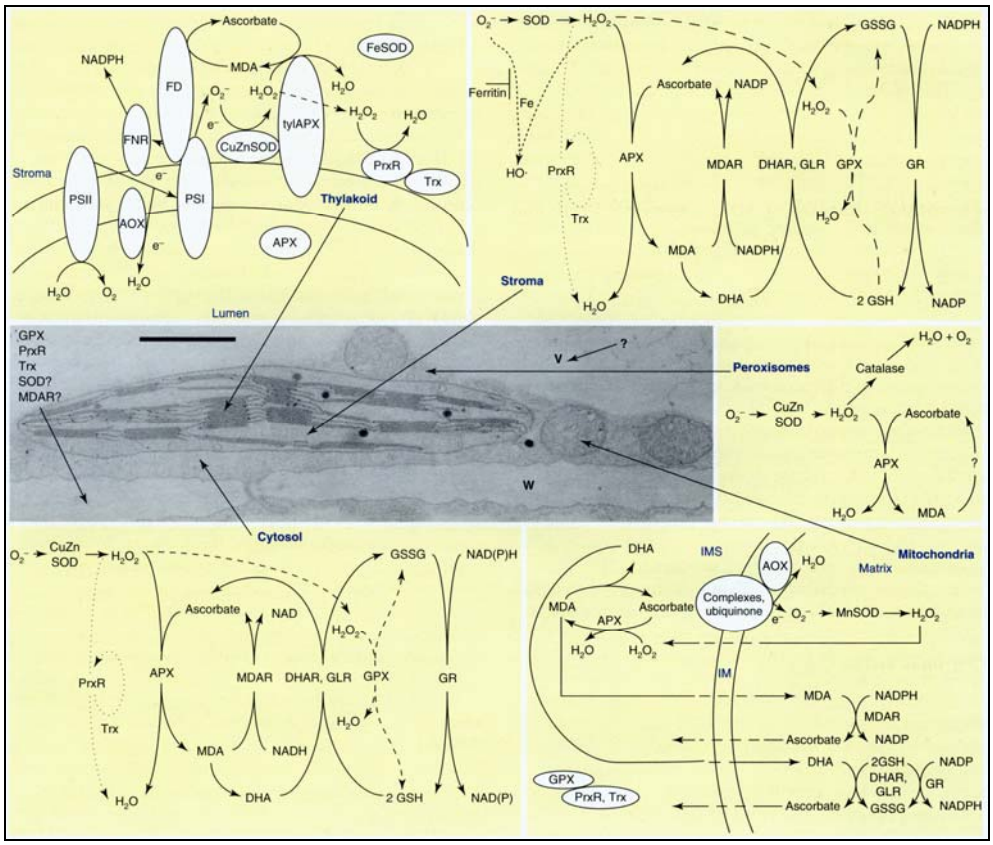


Figure 1.7 *Localization of reactive oxygen species (ROS) scavenging pathways in plant cells (source: Mittler et al., 2004). A transmission electron micrograph of a portion of a plant cell is used to demonstrate the relative volumes of the different cellular compartments and their physical separation (middle left). The enzymatic pathways responsible for ROS detoxification are shown. Membrane-bound enzymes are depicted in white, GPX pathways are indicated by dashed lines and PrxR pathways are indicated by dotted lines in the stroma and cytosol. Abbreviations: AOX, alternative oxidase; APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; glutathione peroxidase (GPX); GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; MDA, monodehydroascorbate; MDAR, MDA reductase; PrxR, Peroxiredoxin; PSI, photosystem I; PSII, photosystem II; SOD, superoxide dismutase; Trx, thioredoxin; tyl, thylakoid; V, vacuole; W, cell wall.*

Furthermore, the ascorbate-glutathione cycle plays a central role in the antioxidant defence mechanism in plant cells (Foyer and Noctor 2003, Foyer and

Noctor 2005a). Antioxidants such as AsA and GSH are found at very high concentrations in chloroplasts and other cellular compartments, and mutants with suppressed AsA levels are more sensitive to pathogenic attack and abiotic stress. The reduction of H_2O_2 by ascorbate can occur directly or it can be catalyzed by ascorbate peroxidase. Then, the oxidized form of ascorbate can be reduced enzymatically by dehydroascorbate reductase using glutathione as an electron donor which in turn is reduced by glutathione reductase in the presence of NAD(P)H.

1.4.3 ROS and regulation

Reactive oxygen species are capable of modulating signalling networks that control physiological processes and stress responses (Mittler *et al.* 2004, Foyer and Noctor 2005a). ROS, such as H_2O_2, are ideal signalling molecules as they are small and able to diffuse over short distances. They can influence the expression of a number of genes involved in signal transduction, metabolism, cellular organization, cell rescue, … (Allen and Tresini 2000, Desikan *et al.* 2001, Gadjev *et al.* 2006). Kacperska (2004) describes possible ways in signal sensing engaged in plant responses to various abiotic stresses, suggesting a complex network depending on the intensity of the stressor. Multiple studies emphasize the existence of extensive cross talk between different signal transduction pathways in plant cells (Knight and Knight 2001, Mithofer *et al.* 2004). Because hydrogen peroxide (H_2O_2) production is an immediate response to increased Cd or Cu, it is probably a key molecule that can trigger signal transduction events after plant metal exposure, mediating the acquisition of tolerance (Bhattacharjee 2005, Maksymiec 2007, Maksymiec *et al.* 2007).

ROS produced in different cellular compartments can be detected by several cellular components such as ROS receptors and redox-sensitive transcription factors (Kovtun *et al.* 2000, Neill *et al.* 2002, Mittler *et al.* 2004). Detection of ROS by receptors can result in the release of Ca^{2+} from intracellular stores or in the activation of phospholipases (Bhattacharjee 2005, Wang *et al.* 2006). The generation of ROS, Ca^{2+} signals and the activation of specific phospholipases are thought to activate Ca^{2+}-dependent kinases as well as other signal transduction cascades including the MAPK (mitogen-activated protein kinase) - pathway (Reddy 2001, Mittler *et al.* 2004). A given ROS can also result in changes in

transcriptional activity by modifying a redox-sensitive transcription factor directly (Laloi *et al.* 2004).

1.4.3.1 MAPK signalling

MAPkinases are one of the largest families of serine-threonine kinases in higher plants that transduce extracellular signals to regulate cellular processes such as cell division, hormone production and defence mechanisms (Zhang and Klessig 2001, Nakagami *et al.* 2005, Mishra *et al.* 2006). They mediate signal transduction from cell surface to the nucleus. During this process of intracellular communication, MAPKs interact with upstream mediators, including growth factor receptors, G-proteins, tyrosine kinases, and downstream mediators, such as nuclear transcription factors. The activation of specific transcription factors induces, in its turn, the expression of specific genes. This way, components of the ROS scavenging network can be induced or a positive amplification loop can be activated resulting in an increased ROS production via NADPH oxidases (figure 1.8) (Mittler *et al.* 2004).

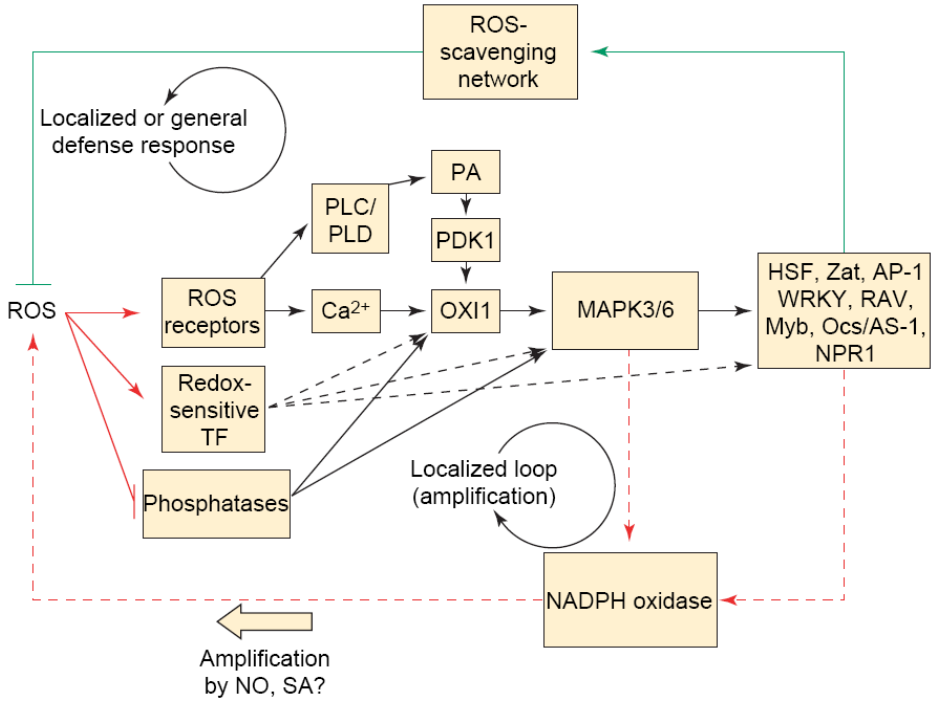


Figure 1.8: *Generalized model of the ROS signal transduction pathway (source: Mittler et al., 2004). Abbreviations: HSF: heat shock factor; OXI1: oxidative signal inducible kinase 1; PA: phosphatidic acid; PDK: phosphoinositide-dependent kinase; PLC/PLD: phospholipase C/D; TF: transcription factor.*

There is increasing evidence that plants rapidly activate MAPKs when exposed to multiple abiotic stress stimuli (Kaur and Gupta 2005). In *Arabidopsis*, H_2O_2 activates MPK3 and MPK6 via the MAPK kinase kinase ANP1 (Kovtun *et al.* 2000, Pitzschke *et al.* 2006), and also OXI1 has been shown to play a central role in ROS sensing and the activation of MAPKs 3 and 6 (Rentel *et al.* 2004).
Also heavy metals can activate MAPKs in higher plants. Both Cd and Cu provoke enhanced ROS production, albeit via distinct underlying pathways, each leading to MAPK activation (Yeh *et al.* 2007). Information regarding specific differences in MAPK induction, however, remains rather fragmentary. In the roots of Cd-exposed plants, a more delayed MAPK activation was detected as compared to the roots of Cu-exposed plants (Jonak *et al.* 2004).

1.4.3.2 Ca^{2+} signalling

Abiotic stresses result in transient increases in cytosolic Ca^{2+}, either to influx from the apoplastic space or release from the internal stores (ER, vacuoles, mitochondria, chloroplasts and nucleus). The precise kinetics, magnitude and cellular source of stimulus-induced $[Ca^{2+}]_{cyt}$ elevations have been proposed to encode information about the particular stimulus, and to determine the specific end response elicited (Reddy 2001). Former studies indicated that different signals use distinct Ca^{2+} sources to elevate $[Ca^{2+}]_{cyt}$ (Knight 2000). Transient increase in the cytoplasmic Ca concentration in response to signals is sensed by an array of Ca^{2+} sensors, such as calmodulin (CaM), CaM-like and other Ca^{2+}-binding proteins, and Ca^{2+}-regulated protein kinases (Reddy 2001). Once Ca^{2+} sensors decode the elevated $[Ca^{2+}]_{cyt}$, Ca^{2+} levels are restored via Ca^{2+} efflux into the cell exterior and/or sequestration in organelles.
Previous research already demonstrated a coordinated link between the redox and Ca^{2+} signalling, and H_2O_2 causes Ca^{2+} peaks by different mechanisms in roots and shoots (Rentel and Knight 2004). Cadmium also influences the cytosolic Ca^{2+}-concentrations, whether or not via H_2O_2 as an intermediate signalling molecule or as a second messenger produced via a Ca^{2+}-induced oxidative burst (Garnier *et al.* 2006). Some studies show evidence for Ca^{2+} acting upstream from ROS (Grant *et al.* 2000), whereas other treatments demonstrate a ROS burst prior to Ca^{2+}-increases, indicating a downstream role (Rentel and Knight 2004). In the case of Cd toxicity, Garnier and collegues

(2006) demonstrated the accumulation of H_2O_2 was preceded by an increase in cytosolic Ca^{2+}, essential to activate NADPH oxidases in BY-2 cells.

1.5 Conclusion

Anthropogenic Cd and Cu contamination is a worldwide problem. In Belgium, especially Cd pollution is of great concern and historical emissions from the non-ferrous metal industry have contaminated the Meuse valley near Liège and the northern part of the Kempen with Cd. Although the overall phytotoxic response of heavy metals is similar, it is of great importance to better understand the underlying molecular mechanisms. This information is most useful to develop or adjust strategies for growing non-food crops on heavy metal-contaminated agricultural soils, whether or not aiming phytoremediation. Moreover, when exploring the fundamental principles of cellular metal stress, differences in the formation, order and specificity of the cellular events can lead to the discovery of metal-specific responses, and may be used as potential biomarkers.

Chapter 2

OBJECTIVES

Human activities often have a negative impact on the natural environment. Heavy metals such as cadmium (Cd) and copper (Cu) can disturb different aspects of the ecosystem which results in great losses of crop production and plant diversity. Physiological effects of these elements were extensively studied in the past. Common responses of plants grown on polluted soil include growth reduction and disruption of physiological processes such as photosynthesis and transpiration. At the cellular level, both metals induce oxidative stress, although knowledge regarding the underlying molecular mechanisms is rather scarce.

Oxidative stress is a common process in multiple stress situations. As the term indicates, it often implies a harmful process, but recent studies showed a biological meaningful role for reactive oxygen species (ROS) in processes such as signal transduction. It is a dynamic process and different stress condititions may result in specific oxidative stress signatures. Consequently, important questions arise towards a specific role for this redox disequilibrium during heavy metal stress.

The main objective of this work concerns the cellular basis of heavy metal-induced responses. It addresses the question to what extent oxidative stress is a negative consequence of heavy metal toxicity or rather a mechanism that triggers specific signal transduction pathways and hence accompanying cellular responses. Additionally, as organisms are mostly exposed in a multipollution context, a better insight in metal-specific (oxidative) stress responses was aimed in this study.

The outline of this thesis includes three main topics:

1. **The study of underlying transcriptional and enzymatic alterations related to oxidative stress during Cd and Cu toxicity.** **Chapter 4** comprises the underlying molecular mechanisms in both roots and shoots under Cd stress, and Cd-induced responses were compared with Cu-induced cellular mechanisms in **chapter 5**. Specific monometallically

induced effects are discussed in **chapter 6**, in which Cd and Cu induced responses were also studied in a multipollution context.

2. The results of part 1 indicated treatment- and organ-characteristic responses, which point towards the existence of specific underlying regulation mechanisms. **In part 2 metal-specific oxidative stress signatures were further unravelled and expanded to the study of different signal transduction cascades**. To gain better insight in the molecular mechanisms behind Cd stress, a transcriptional screening at the root level was performed in **chapter 7**. Based on these results, the induction of signal transduction cascades was studied in the roots and leaves under Cd (**chapters 7 and 8**) and Cu stress (**chapter 9**). The role of signalling components such as MAPkinases, Ca^{2+}-dependent kinases and miRNAs was further explored and metal-specific responses were revealed.
3. **Chapter 10** was dedicated to the **role of OXI1** (oxidative signal inducible kinase) during heavy metal stress, an important outcome of the previous chapters.

Chapter 3

MATERIALS AND METHODS

PART I: Optimalisation of a hydroponic set-up for *Arabidopsis thaliana*

Karen Smeets, Joske Ruytinx, Frank Van Belleghem, Brahim Semane, Dan Lin, Jaco Vangronsveld, Ann Cuypers (2008). Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. Plant Physiology and Biochemistry. 46 (2), 212-218.

Abstract

Arabidopsis thaliana is one of the most widely used model organisms in plant sciences. Because of the increasing knowledge in the understanding of its molecular pathways, a reproducible and stable growth set-up for obtaining uniform plants becomes more important. In order to be able to easily harvest and study both roots and shoots, and to allow simple exposure to water-soluble toxic substances, a hydroponic system is the desired cultivation method for controlled plant growth. Based on earlier developed hydroponic cultivation protocols, a hydroponic set-up was optimized and statistically validated using linear mixed-effects models. In order to determine important components that influence the level of variability in a hydroponic set-up, stress-related indicators were examined on the biochemical as well as on the molecular level. It is highly recommended that statistical as well as biological assumptions are carried out before post-analyses are performed. Therefore, we suggest a model where factors that influence variability such as the usage of different pots and harvesting on different times are taken into account in the analyses. Furthermore, in contrast to what has been reported in earlier studies, our findings indicate that continuous aeration of the hydroponic solution is highly important.

3.1.1 Introduction

Arabidopsis thaliana is one of the most widely used model organisms in plant sciences. Particularly in molecular research, *Arabidopsis* is of great interest and its widely available genetic information makes several molecular techniques less problematic to work with (Schenk *et al.* 2000, Jung *et al.* 2003, Dean 2004). It is a very useful and popular species for studying gene function and regulation and for investigating (new) genetic pathways in normal conditions but also in comparison with several stress situations. Furthermore, *Arabidopsis* is relatively easy to grow and transform and multiple mutants are available.

Using *Arabidopsis* as a model organism makes it possible to study contemporaneously at physiological, biochemical and molecular levels; however, a sufficient amount of biomass is required. On the other hand, from statistical point of view, the individual plant and/or sample variation has to be minimized. It is highly recommended to prevent variation in the controls induced by external factors. Gene expression is one of the most sensitive parameters and even small external variations can cause high variability in the transcriptome (Vansuyt *et al.* 1997, Singh *et al.* 2002, Li *et al.* 2005).

Several protocols have been described for the hydroponic cultivation of *Arabidopsis thaliana* (Gibeaut *et al.* 1997, Arteca and Arteca 2000, Huttner and Bar-Zvi 2003, Schlesier *et al.* 2003, Tocquin *et al.* 2003, Noren *et al.* 2004, Heidenreich *et al.* 2005, Robison *et al.* 2006). We combined some of these protocols to obtain a large-scale system with a high uniformity of plant material. In order to determine important components that influence the level of variability in a hydroponic set-up, stress-related indicators such as parameters of the antioxidative defence system were examined on the biochemical as well as on the molecular level. The level of variability and the reproducibility of the set-up were statistically evaluated using linear mixed-effects models. Furthermore, the need for aeration, even when plants are harvested in a quite young stage, was statistically evaluated in this cultivation system.

3.1.2 Materials and methods

3.1.2.1 Hydroponic cultivation

As plant material, *Arabidopsis thaliana* (Columbia ecotype) was used. The seeds were surface sterilized and, in order to synchronize the germination, dark incubated during 3 days at 4 °C on a filter paper soaken with tap water.

Based on previous studies (Gibeaut *et al.* 1997, Huttner and Bar-Zvi 2003), a hydroponic system was developed to grow *Arabidopsis* plants simultaneously with a small individual variation.

3.1.2.1.1 Set-up

The bottom part (+/- 9 cm) from 15 ml polyethylene centrifuge tubes was removed and rockwool pieces of 2 cm by 1.5 cm were placed in the remaining part of the tubes (figure 3.1). The rockwool-holding plugs were positioned in test tube racks that, for their part, were placed in aluminium-covered pots filled with nutrient solution (figure 3.2). The nutrient solution was based on Hoagland growth medium. Nutrient concentrations were tested and optimised: 0.505 mM KNO_3, 0.15 mM $Ca(NO_3)_2.4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.1 mM $MgSO_4.7H_2O$, 4.63 µM H_3BO_3, 0.91 µM $MnCl_2.4\ H_2O$, 0.03 µM $CuSO_4.5H_2O$, 0.06 µM $H_2MoO_4.H_2O$, 0.16 µM $ZnSO_4.7H_2O$, 1.64 µM $FeSO_4.7H_2O$ and 0.81 µM Na_2-EDTA. A few seeds were placed on top of each rockwool plug. The plugs were pre-moistened with the nutrient solution. To simplify the seed placement, seeds were brought in water and pipetted onto the plugs. A 12-h photoperiod at 65% relative humidity and day/night temperatures of 22 °C and 18 °C were used. Light was supplied by cool white fluorescent lamps (L 140W/20SA, Osram, Ausburg) at a photosynthetic photon flux density of 165 µmol $m^{-2}s^{-1}$ at the leaf level above all pots. During the first three days of germination, pots were covered with glass plates to prevent dehydration of the rockwool and to obtain an even more controllable and stable growth situation.

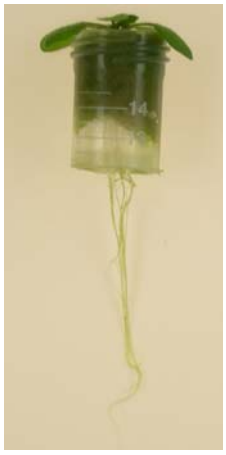
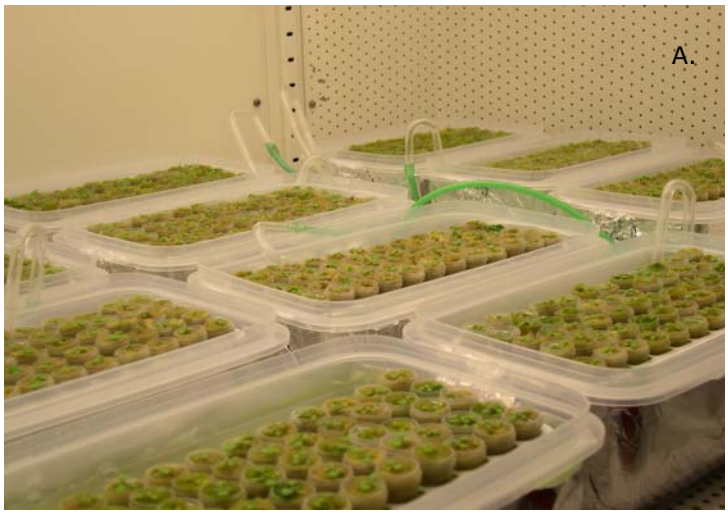


Figure 3.1: *Set-up of an individual rockwool-holding tube. Rockwool pieces of 2 cm by 1.5 cm are placed in the upper part of a 15 ml polyethylene centrifuge tube, as an inert support for the cultivated plant.*

The nutrient solution was refreshed every 3 days based on the changes in pH- and conductivity. After one week of germination, plants were thinned to one plant per plug; the nutrient solution was continuously aerated and laterally covered with polystyrene plates.



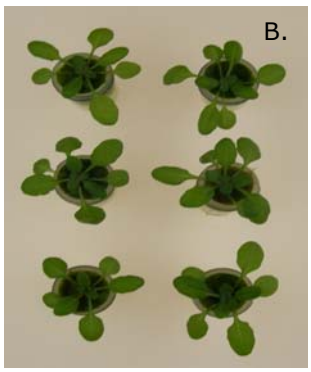


***Figure 3.2**: A. Set-up of the cultivation system. The rockwool-holding tubes are positioned in test tube racks that are placed in aluminium-covered pots filled with nutrient solution. The pots are continuously aerated. B+C Arabidopsis plants after 3 weeks of growth. Morphologically, no visual differences are noticed.*

3.1.2.1.2 Harvesting

At harvesting (after 3 weeks), morphological parameters were determined and leaf samples for the molecular and biochemical analyses were collected. For the first experiment three biological samples per pot were taken with each sample weight close to 100 mg. For the kinetic experiment, three biological replicates were taken after 0h, 2h, 4h, 8h and 24h. For the aeration experiment, 5 biological replicates were taken after 1 week without aeration, and compared with samples where the solution was continuously aerated.

All samples were frozen in liquid nitrogen and stored at -80°C.

3.1.2.2 Analysis of enzyme activities

Frozen leaf tissue was homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone (1 ml buffer 100 mg^{-1} fresh weight). The homogenate was squeezed through a nylon mesh and centrifuged for 10 minutes at 20000 g and 4°C. The enzyme activities were measured spectrophotometrically in the supernatant at 25°C.

Analysis of superoxide dismutase (SOD, EC 1.15.1.1) activity was based on the inhibition of cytochrome c at 550 nm (McCord and Fridovich 1969). Guaiacol peroxidase and catalase activities (GPX, EC 1.11.1.7; CAT, EC 1.11.1.6) were determined at 436 nm and 240 nm respectively according to Bergmeyer (Bergmeyer *et al.* 1974). Analysis of glutathione reductase (GR, EC 1.6.4.2) activity was based on the reduction of GSSG (340 nm), using NADPH as described by Bergmeyer (Bergmeyer *et al.* 1974).

3.1.2.3 Analysis of gene expression

Frozen leaf tissue (100 mg) was ground thoroughly in liquid nitrogen using a mortar and pestle. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). The concentration of the RNA was determined spectrophotometrically at 260 nm (Nanodrop, Isogen Life sciences). The RNA-purity was also checked spectrophotometrically by means of the 260/280-ratio. First strand cDNA synthesis was primed with an oligo$(dT)_{16}$-primer according to the manufacturer's instructions using Taqman Reverse Transcription Reagents (Applied Biosystems). Quantitative PCR was performed using equal amounts of cDNA with the ABI Prism 7000 (Applied Biosystems), Taqman chemistry. Assay mix (primers and probes) was optimised by Assays-by-design (Applied Biosystems). PCR amplifications were performed in a total volume of 25 µl; containing 5 µl cDNA sample, 12.5 µl Taqman Universal Master Mix (Applied Biosystems), 1.25 µl assay mix (Applied Biosystems) and 6.25 µl RNase-free H_2O. All samples were tested in duplicate or triplicate, for the housekeeping gene as well as for the genes of interest. Following genes were determined: catalase 1 (*CAT1*: At1g20650), glutatathione reductase 1 (*GR1*: At3g24170), alcohol dehydrogenase 1 (*ADH1*: At1g77120), lactate dehydrogenase 1 (*LDH1*: At4g17260) and pyruvate decarboxylase 1 (*PDC1*: At4g33070). As a reference gene actine 2 (*ACT2*: At3g18780) was used.

The efficiencies of the target genes related to the reference gene were examined and approved. Gene expression data were calculated relatively to the reference gene following the $2^{-\Delta\Delta Ct}$ – method (Livak and Schmittgen 2001).

3.1.2.4 Statistical analysis

In order to determine if there occurred a significant difference between the enzyme activities and gene expression measurements of the different pots, both datasets (gene expression and enzyme activities) were analyzed using linear mixed-effects models (Verbeke and Molenberghs 2000). Transformations were applied when necessary to approximate normality. The random pot effect was specified in the model to estimate the variability between pots. The model also took into account the heterogeneous variability for each pot. The nutrient datasets were also analyzed using linear mixed-effects models. The Dunnett multiple comparison adjustment was applied to obtain corrected *p*-values.
Furthermore, the time effect of the genes was examined and analyzed by means of a regression model, whereas for the aeration experiment a t-test was used (Neter *et al.* 1996). Transformations were applied on both datasets when necessary to approximate normality. All statistical analyses were performed using SAS 9.1.

3.1.3 Results and discussion

The aim of this study was to obtain and evaluate an experimental set-up for growing, harvesting and analyzing *Arabidopsis thaliana* plants, based on several known protocols (Gibeaut *et al.* 1997, Huttner and Bar-Zvi 2003, Noren *et al.* 2004, Robison *et al.* 2006). When molecular parameters are investigated, not only the uniformity of the plant material is important, but also the choice of set-up and the way of sampling are key parameters for reducing the variability between different samples. Whereas in most of the previous studies morphological parameters such as biomass were tested (Gibeaut *et al.* 1997, Arteca and Arteca 2000, Noren *et al.* 2004, Robison *et al.* 2006), we decided to use more sensitive molecular and biochemical characteristics to test the reproducibility and the homogeneity of a hydroponic system (Gibeaut *et al.* 1997, Huttner and Bar-Zvi 2003, Noren *et al.* 2004, Robison *et al.* 2006). The experimental set-up of the entire hydroponically growth system for *Arabidopsis*

seedlings is presented in figure 3.2A. Plants were cultivated under similar conditions in the same growth chamber.

3.1.3.1 Important variability parameters

In a first experiment, several parameters (gene expression and enzyme activities of antioxidative mechanisms) were measured in sixteen different pots. The parameters chosen are stress-related and are rapidly induced by variations of external conditions.

After 3 weeks, weight of the roots was 25.78 ± 3.8 mg and the upper ground parts consisted of ± 10 leaves with an average weight of 54.66 ± 6.62 mg. Figure 2B is illustrative for the high homogeneity between the different plants in distinct pots. A time period of three weeks was chosen because from statistical point of view, it is better to minimize the individual (plant) variation. With high individual variability, some of the stress effects will be masked. Since *Arabidopsis* is a rosette plant, especially older plants (more biomass) are more variable because of the large differences in the physiological age and status of the leaves (Brown *et al.* 2003, Panchuk *et al.* 2005). In this perspective it is interesting to take several (younger) small plants of the same physiological level (and hence equal cellular homeostasis and redox status). For that reason, a cultivation method was chosen that allowed to grow high numbers of plants in the same area (figure 3.2). In this way it is also possible to test more conditions in the same culture, which also decreases the overall variability.

To make sure that the experimental set-up is suitable for molecular-biochemical studies on plants exposed to mild stresses, it is necessary that the steady-state level of the control plants is reproducible between the replicas in one experiment. Therefore we analyzed some components of the antioxidative defence system at 2 different biological organization levels: transcription level (gene expression) on one hand and biochemical level (enzyme activities) on the other hand.

In our set-up, changes in activities of catalase (CAT), guaiacol peroxidase (GPX) and glutathione reductase (GR) were analyzed in the leaves. Generally, activities of both, CAT as well as GPX, are quite rapidly increased when exposed to (mild) stress situations (Ali *et al.* 2005a, Smeets *et al.* 2005). Especially catalases are central components in maintaining the cellular redox balans and therefore relatively sensible to external variations (McClung 1997, Orendi *et al.* 2001). GR

is an important enzyme in the maintenance of the reduced state of glutathione, which is a key antioxidative metabolite with high regulatory properties in stress situations (Xiang and Oliver 1998). The null hypothesis (=no differences between the pots) was accepted when $p>0.05$. The H_2O_2-quenching enzymes such as catalase and guaiacol peroxidase showed no significant differences in activities between the sixteen different pots of the system, with p-values of 0.9977 and 0.7912 respectively (table 3.1). In addition, we can conclude from the model that there was no significant difference in mean GR activity, p-value = 0.0514. In each of the 16 different pots, however, differences in within-pot variability and correlation coefficients were detected, which means samples taken from the same pot are more correlated (table 3.1).

***Table 3.1**: Enzyme activities in the leaves of Arabidopsis thaliana cultivated in different pots. Each value represents the mean ± S.E. of three determinations. No significant differences were found between the pots at significance level 0.05. The within pot correlation coefficients are given between parentheses.*

Pot	GPX (mU/g FW)	GR (mU/g FW)	CAT (mU/g FW)
1	983,80 ± 128,52 (0.19)	76,17 ± 1.34 (0.11)	619,63 ± 121.89 (0.05)
2	841,50 ± 207,48 (0.08)	100,47 ± 6.85 (0.006)	555,74 ± 111.37 (0.04)
3	1210,6 ± 170,53 (0.18)	99,67 ± 16.93 (0.001)	757,46 ± 52.16 (0.36)
4	1424,85 ± 83,66 (0.47)	101,41 ± 4.62 (0.23)	663,98 ± 8.40 (0.93)
5	1138,69 ± 64,2 (0.56)	88,89 ± 5.36 (0.008)	745,42 ± 96.75 (0.13)
6	1128,1 ± 201,71 (0.09)	98,84 ± 7.00 (0.006)	675,11 ± 96.49 (0.08)
7	1046,64 ± 161,9 (0.13)	102,26 ± 7.55 (0.01)	724,99 ± 13.94 (0.86)
8	925,71 ± 48,13 (0.56)	85,09 ± 11.38 (0.002)	615,71 ± 22.34 (0.6)
9	1170,64 ± 189,8 (0.43)	93,97 ± 10.50 (0.003)	674,25 ± 103.68 (0.08)
10	943,89 ± 50,22 (0.59)	90,99 ± 5.65 (0.014)	687,22 ± 30.07(0.53)
11	1244,97 ± 181,4 (0.14)	99,76 ± 14.08 (0.002)	639,98 ± 53.51 (0.23)
12	889,97 ± 171,69 (0.09)	90,45 ± 6.04 (0.009)	540,07 ± 109.02 (0.04)
13	1120,47 ± 171,8 (0.17)	114,54 ± 6.57 (0.01)	603,45 ± 94.77 (0.12)
14	900,92 ± 103,15 (0.25)	100,79 ± 4.72 (0.019)	606,52 ± 94.14 (0.07)
15	1324,27 ± 288,4 (0.08)	113,12 ± 13.89 (0.002)	757,71 ± 55.03 (0.32)
16	762,84 ± 83,89 (0.29)	105,27 ± 7.76 (0.01)	728,16 ± 13.08 (0.89)

At transcriptional level, the gene expression level of catalase1 *(CAT1)* and glutathione reductase1 *(GR1)* was measured in the leaves. Considering the gene expression of *CAT1* the gene expression values fluctuated around 4.6 ± 0.9 (ΔCt values, normalized to the level of actin). The gene expression level of *GR1* had an average of 4.6 with a standard error of 0.8 (ΔCt values, normalized to the level of actin). A hypothesis test was performed to determine whether the mean difference in gene expression between the gene of interest and the housekeeping gene varied over the 16 pots. Both for *CAT1* (p-value = 0.4266) and *GR1* (p-value = 0.4291), normalized to the housekeeping gene, there was no significant pot effect found. Also on gene expression level, however, differences in within-pot variability and correlation were detected.

Our results indicate that the proposed hydroponic system leads relatively stable and constant parameters on the molecular-biochemical level and should deliver reproducible overall results. Nevertheless, although no differences were observed between the means of the different pots, the within pot variability did vary over the 16 pots. This is an important factor that has to be taken into account when setting up an experiment or harvesting the plant tissues. We propose a growth system were multiple pots are used per treatment/condition so that samples of different pots are included in the analysis. We advice to take, for each parameter, multiple samples per pot, and this for at least two pots. As such, the within-pot correlation can be taken into account in the analyses (correction of pot effect), and because samples from different pots were taken, the outcome is not dependent on the pot effect, but on the treatment effect. Furthermore, when using multiple pots per treatment, randomization can improve the experimental design.

3.1.3.2 Composition of Hoagland nutrient solution

The nutrient solution was based on Hoagland growth medium. Many genes are transcriptionally responsive to changes in nutrient availability and nutrient concentrations were tested and optimized (Salt 2004). Following nutrient concentrations were used: 0.505 mM KNO_3, 0.15 mM $Ca(NO_3)_2.4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.1 mM $MgSO_4.7H_2O$, 4.63 µM H_3BO_3, 0.91 µM $MnCl_2.4\ H_2O$, 0.03 µM $CuSO_4.5H_2O$, 0.06 µM $H_2MoO_4.H_2O$, 0.16 µM $ZnSO_4.7H_2O$, 1.64 µM $FeSO_4.7H_2O$ and 0.81 µM Na_2-EDTA. The corresponding root and shoot concentrations are describe in table 3.2A. The pH and conductivity level of the nutrient solution was

checked every day. Mainly during the last two weeks, the pH level increased from 5.75 to 6.22 ± 0.04 whereas the conductivity level decreased from 195.32 ± 7.07 to 127 ± 3.07 in just a few days. According to these values we decided to renew the solution every three days, in contrast with most of the previous studies where the medium was changed weekly.

Element	**Root** (mg kg^{-1} DW)	**Leaf** (mg kg^{-1} DW)
Na	1466±167	1495±214
Mg	4199±731	9626±186
P	11480±421	10281±231
S	5837±795	9407±260
K	31824±1824	28121±687
Ca	9864±1792	37351±695
Mn	570±110	207±9
Fe	5097±512	594±121
Cu	21±2	12±1
Zn	221±15	101±5

Table 3.2:

A. Element concentration (mg kg^{-1} DW) of 3-week-old Arabidopsis thaliana seedlings. Values are mean ± SE of 4 independent biological replicates

B. Element concentration (mg kg^{-1} DW) of 3-week-old Arabidopsis thaliana seedlings grown in Hoagland solution with no chelate, EDTA, 1:10 EDTA or tartrate. Values are relatively expressed to the control=100% (= Hoagland with EDTA). Values are mean ± SE of 3 independent biological replicates.

Element	**Root** (mg kg^{-1} DW)			**Leaf** (mg kg^{-1} DW)		
	no chelate	1:10 EDTA	tartrate	no chelate	1:10 EDTA	tartrate
Ca	98,83±36	68,09±33	125,74±17	153,19±10	113,09±8	139,4±17
Mn	106,27±36	59,84±3	113,84±16	170,29±36	56,08±6	144,11±27
Fe	105,35±33	35,29±2	131,79±13	254,22±180	73,88±38	160,68±112
Zn	120,45±12	139,84±22	93,97±9	139,49±13	1083±13	122,19±8

Furthermore, because of the known effects of EDTA on the solubility of several elements, another experiment was carried out to test the effect of EDTA on the accumulation of different nutrients (Sarret *et al.* 2001, Lai and Chen 2004). Plants were grown under the same circumstances as mentioned above, but the use of EDTA as a chelate was compared with the use of diluted EDTA, tartrate or

the use of no chelate at all. Differences in the root and shoot accumulation of the elements Ca, Mn, Fe and Zn are represented in table 3.2B. In the shoots, the use of tartrate or the use of no chelate resulted in higher element concentrations and more variability between the biological replicates of Mn, Fe and Zn. In the roots, no clear differences were found, except for the $1/10^{th}$ dilution of EDTA. In both root and shoot samples, more variability between the biological replicates was detected when no chelate or tartrate were used. Therefore, we concluded the addition of EDTA leads the most stable and representative results as compared to the literature.

3.1.3.3 Aeration

In previous studies regarding to the aeration of the root zone, it was stated that lack of aeration had no effect on the root and shoot weight of the plants (Arteca and Arteca 2000, Robison *et al.* 2006). Because of the growing importance of molecular research, we tested the effect of aeration on the gene expression level of genes that are induced under hypoxia, in combination with the activities of antioxidative enzymes which are activated in stress situations (Dennis *et al.* 2000, Bailey-Serres and Chang 2005, Branco-Price *et al.* 2005). In addition, the oxygen levels were determined in the nutrient solutions with and without aeration. Our results show an oxygen depletion level from 88% to 11% (as compared to the open air) during three days, which means a reduction in oxygen concentration from 7.9 ± 1.2 mg/L to 1.0 ± 0.3 mg/L. In the aerated plots the oxygen level remained stable (± 8 mg/L).

This oxygen depletion appeared to induce a strong effect on gene expression level. Highly significant transcriptional inductions of *ADH1* (alcohol dehydrogenase) and *PDC1* (pyruvate decarboxylase) were observed in the roots when no aeration was applied in the medium (figure 3.3). In the leaves of plants without aeration to the root zone, *LDH1* (lactate dehydrogenase) was significantly upregulated (figure 3.3). Only a subpopulation of hypoxia-induced genes was tested, but we might suspect more transcriptional changes due to gene interactions and gene network regulation. Futhermore, the effect of aeration on the activities of superoxide dismutase, catalase and guiacol peroxidase was measured. Especially guiacol peroxidase activity was affected. In the roots, a 6-fold increase in activity level was observed when no aeration was applied to the medium. In the leaves, guiacol peroxidase activity decreased with

a factor 12 in the plants without aeration (table 3.3). According to these results, we strongly advise a continuous aeration of the nutrient solution.

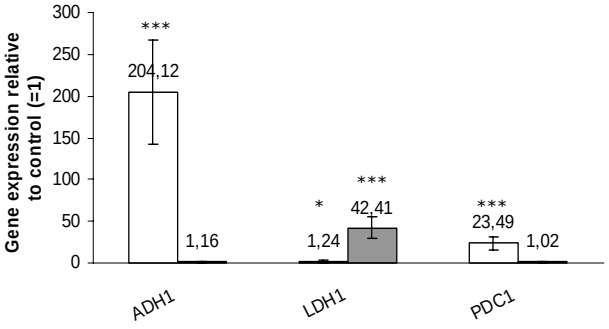


***Figure 3.3**: Gene expression level of three hypoxia-induced genes. The gene expression levels are expressed in function of the control values (= aerated Arabidopsis plants) which are equal to 1. Each point represents the mean ± S.E. of five determinations in roots (white) and leaves (grey) of Arabidopsis plants without aeration to the root zone (significance levels : *: p<0.1; **: p<0.05; ***: p<0.01)*

***Table 3.3**: Enzyme activities (mU/g FW) in the leaves of Arabidopsis thaliana cultivated with and without aeration to the root zone. Each value represents the mean ± S.E. of four determinations (significance levels : *: p-value<0.1; **: p-value<0.05; ***: p-value<0.01).*

	ROOTS		LEAVES	
Enzymes	With aeration	Without aeration	With aeration	Without aeration
SOD	275684±31726	241497±27845	137844±9024	100152±18422*
GPX	6085±1038	1003±52***	837±135	11113±1974***
CAT	49±2	41±5	204±24	226±10

3.1.3.4 Kinetic experiment

At harvesting, samples of different treatments were often taken at different daytimes. From statistical point of view, time is an important factor which induces heterogeneity between samples. Therefore, the constancy of the gene expression was tested over time (figure 3.4). *CAT1* and *GR1* were analyzed on gene expression level during 24h. Measurements were performed on samples taken in the morning (9 am and 11 am), at 1 pm, at 5 pm and again at 9 am the next morning. For *CAT1*, a time effect to the 3rd power was observed with a p-value of 0.0041. *GR1* showed a time effect to the 2nd power (p-value < 0.0001), with a high expression in the morning. Also other genes seem to fluctuate during the course of the day. In this experiment for example,

glutathione peroxidase2 or manganese superoxide dismutase1 showed time effects to the 3rd power with p-values < 0.0001 (figure 3.4). On the other hand, genes like iron superoxide dismutase1 remained stable and do not seem to be influenced at all (p-value = 0.898) (results not shown).

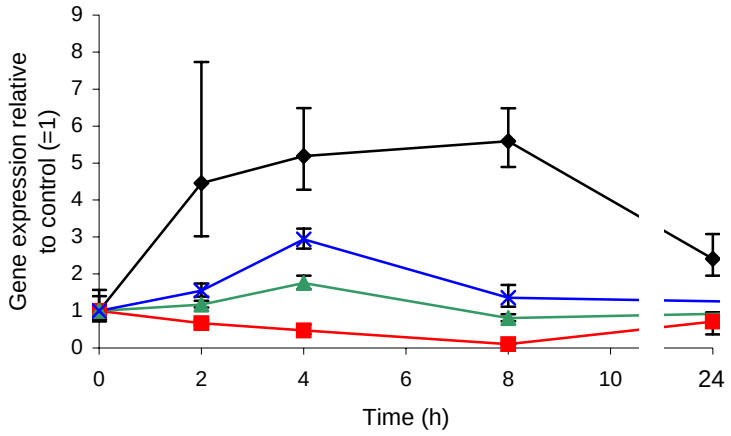


Figure 4: Time effects in gene expression level of four antioxidative genes: *CAT1* (◆), *GR1* (■), *GPX2* (▲) and *MSD1* (×). The gene expression levels are expressed in function of the control values (= harvested at time zero) which are equal to 1. Each point represents the mean ± S.E. of six determinations on different time periods. Significant time effects were noticed in each of the represented genes (signicance level = 0.05).

Therefore, due to time-related effects, sampling within the same time period of control and treated samples is essential to keep variability under control. It is of great importance, especially when studying transcriptional alterations, that the measured treatment effects will not be masked by circadian rhythms of the plant. Many genes are regulated by the light regime of the day, or are expressed according to a circadian rhythm. Since results vary significantly in gene expression level between samples taken at less than a few hours of time difference, an appropriate harvesting protocol is required (figure 3.4).

3.1.4 Conclusion

A hydroponic set-up is probably the most suitable cultivation system to obtain optimal and controllable growth circumstances when both root and shoot samples have to be harvested. Nevertheless, in the past, most of the known hydroponic cultivation systems were only tested on the morphological level (biomass, and other morphological or visible parameters, …). Therefore, a

hydroponic cultivation system was evaluated and optimized to test treatment effects on the molecular as well as on the biochemical level. Furthermore, our results indicate that it is important to pay attention to the variations induced by the cultivation system, especially when sensitive parameters such as gene expression are studied. When different treatments have to be compared, we highly recommend an appropriate harvesting protocol to prevent that effects will be time-related or influenced by the within-pot correlation. The latter can be estimated by collecting and analyzing samples from at least two different pots per treatment. It is also important to reduce the variability in each individual sample (more heterogeneity in older plants). Additionally, the cultivation parameters need to remain relatively stable. The nutrient solution should be regularly refreshed and external fluctuations should be avoided. Our results further indicated strong effects of oxygen depletion on the transcriptional level of several hypoxia-related genes. Therefore a continuous aeration of the nutrient solution is suggested.

MATERIALS AND METHODS

PART II: Description of the used techniques

3.2 Plant material and treatment

Arabidopsis thaliana plants (ecotype Columbia) were grown according to the hydroponic set-up, as described previously (3.1). Three-week-old plants were treated with $CdSO_4$ or $CuSO_4$ to the roots and studied after 24h of exposure. At harvesting, the entire aerial part as well as the root system was removed and snap frozen in liquid nitrogen prior to biological measurements.

In chapter 10 *oxi1* mutants with background Wassilewskija were used, because at the start of the experiment, no *oxi1* mutants with background Columbia were available to us.

3.3 Morphological changes and elemental profiles

3.3.1 Electron microscopy

Samples (max 1 mm^2) from roots were fixed using vacuum infiltration for 4 hours at 4 °C in 2 % glutaraldehyde and 0.01 % malachite green, buffered in 0.05 M sodium-PIPES (pH 7.5). The fixed tissues were rinsed 3 times 30 minutes in 0.05 M sodium-PIPES (pH 7.5) and post-fixed in 2% osmium tetroxide, buffered in 0.2 M sodium cacodylate at 4 °C. Subsequently, the tissues were rinsed once in 0.2 M sodium cacodylate and twice in distilled water before staining in 2 % uranyl acetate overnight. After dehydration in a graded acetone series, the tissues were impregnated and embedded in Spurr's epoxy resin. Ultrathin sections (65 nm) were obtained using a Leica Ultracut UCT ultramicrotome and mounted on coated copper grids (50 mesh). The sections were examined using a Philips EM 208S transmission electron microscope operating at 80 kV and digitized with a Morada 3.0 TEM camera controlled by iTEM FEI (version 5.0) software from Olympus Soft Imaging Solutions GmbH. The cell wall thickness of the xylem vessels was measured on digitized sections using iTEM FEI software. Five plants were examined per exposure concentration, with a total of 20-25 measurements per plant.

3.3.2 Element analysis

At the time of harvesting, some of the plants were dried at 80°C and digested in HNO_3 (70-71%) using the heat block. The metal concentration was determined by inductively coupled plasma - atomic emission spectrometry. Blanks (only HNO_3) and a standard sample [NIST Spinach (1570a)] were analyzed for reference purposes. Roots were washed twice for 10 min at 4 °C with 1 mM $Pb(NO_3)_2$ and H_2O_2 to exchange surface-bound elements.

3.4 Reactive oxygen species and lipid peroxidation

3.4.1 Determination of free radicals

Free radical production in the Cd-exposed *Arabidopsis* roots and leaves was measured by post-*in vivo* spin trapping, as described earlier (Hideg and Björn 1996, Hideg and Vass 1996). Briefly, samples were frozen in liquid N_2, then 30 mg aliquots of frozen powder were ground with 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-oyrroline N-oxide) as spin trap (Frejaville *et al.* 1995). This crude extract was filtered and EPR (electron paramagnetic resonance) spectra of the DEPMPO spin adducts were measured at room temperature with the Bruker ECS-106 X-band spectrometer. The time between harvesting and EPR measurement was kept uniform for all samples. To identify DEPMPO spin adducts, EPR spectra detected from sample extracts were compared with simulated model spectra. These were calculated using hyperfine line splitting constants reported as characteristic to specified oxygen radicals.

3.4.2 H_2O_2 measurements

The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes/Invitrogen, Carisbad/California, USA) was used to measure hydrogen peroxide (H_2O_2) levels in both roots and leaves. This is a one-step fluorimetric method that uses 10-acetyl-3,7-dihydroxyphenoxazine to detect H_2O_2. A triplicate of 20 mg root samples was processed for each exposure concentration and measured together with a H_2O_2 standard curve. The fluorescence emission spectrum (590 nm) was measured at an excitation wavelength of 530 nm using

a PTI (Photon Technology International) QuantaMaster Model QM-6/2005 spectrofluorometer equipped with FeliX32 Software & BryteBox Interface.

3.4.3 Determination of lipid peroxidation

The TBA reactive compounds of the plant roots were measured spectrophotometrically to estimate the amount of lipid peroxidation. Plant tissue (200 mg) was homogenized with 3 ml 0.1% TCA buffer and 4 ml 0.5% TBA was added to the extract. After the extract was heated at 95 °C for 30 min and centrifuged for 10 min at 20.000*g*, the absorbance of the supernatant was measured at 532 nm corrected for unspecific absorbance at 600 nm according to Dhindsa et al. (Dhindsa *et al.* 1981).

3.5 Transcriptome analysis

3.5.1 Microarray experiments and data analysis

Frozen root tissue (approximately 100 mg) was ground thoroughly in liquid nitrogen using a mortar and pestle. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). The concentration of the RNA was determined spectrophotometrically at 260 nm (NanoDrop, NanoDrop Technologies). The RNA purity was checked using the Bioanalyzer (Agilent). One µg of each RNA sample was used to synthesize first and second strand cDNA and RNAs were *in vitro* transcribed according to the instruction manual (Amino Allyl MessageAmp II aRNA Amplification Kit, Ambion). For hybridization of labelled aRNAs (4 biological replicates/treatment), microarray slides including >26.000 DNA elements (70-mer gene-specific oligonucleotides; Qiagen/Operon) for known or putative open reading frames were used (printed at the University of Arizona: http://ag.arizona.edu/microarray). After hybridization, slides were scanned (AXON, Genepix4100A) and spot intensities were extracted.

In the first part of the data analysis, genes with missing values were removed and the intensity values were analyzed. The array specific background transformation presented by Van Sanden *et al.* (2006) was applied to the data in order to remove the non-linear effects that were present on the logarithmic scale. Subsequently, an anova model was used to remove array, dye and array-

dye interaction effects shared by the genes (Kerr *et al.* 2000, Van Sanden and Burzykowski 2006).

After this normalization, a Significance Analysis of Microarray (SAM) was performed on both dyes separately (Tusher *et al.* 2001). For each dye, SAM analysis is performed to find genes that differentiate any of the two higher doses from the control (an analogue of the F-test in anova models). SAM is a resampling-based method for significance testing in microarray experiments, while empirically controlling the False Discovery Rate (FDR). FDR is defined as the proportion of false positives among the total number of discoveries. The method is popular for microarray settings because controlling FDR leads to more rejections while accepting a rate of finding false positives. Genes found commonly significant for both dyes are used for further analysis. For these genes, the direction of the expression level with respect to the increasing doses is determined by the sign of their t-test statistics.

3.5.2 Analysis of gene expression

The total RNA sample (extracted using the RNeasy Plant Mini Kit) was briefly incubated in gDNA wipeout buffer at 42 °C for 2 min to effectively remove contaminating gDNA. First strand cDNA synthesis was primed with a combination of oligo(dT)-primers and random hexamers according to the manufacturer's instructions using QuantiTect Reverse Transcription Kit (Qiagen), and equal amounts of starting material (total RNA) were used (1 µg). Quantitative PCR was performed with the ABI Prism 7000 (Applied Biosystems), SYBR green chemistry. Primers were designed and optimized using Primer Express (Applied Biosystems) and the genes with primer sequences are represented in table 3.4. PCR amplifications were performed in a total volume of 20 µl, containing 4 µl cDNA sample, 10 µl Power SYBR green Master Mix (Applied Biosystems), 0.6 µl primers (10 µM) and 5.4 µl RNase-free H_2O. All samples were tested in duplicate for the housekeeping gene as well as for the genes of interest.

The efficiencies of the target genes related to the reference gene were examined and approved. Gene expression data were calculated relative to multiple reference genes: *actin2 (ACT2)*, *At2g28390*, *At3g18780*, *At4g26410, At5g15710* and *At5g80290* (Vandesompele *et al.* 2002, Czechowski *et al.* 2005).

Table 3.4

Gene	Reverse primer	Reverse primer
LOX1 (At1g55020)	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
LOX2 (At3g45140)	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC
RBOHC (At5g51060)	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
RBOHD (At5g47910)	TATGCATCGGAGAGGCTGCT	TAGAGACAACACGTTCCCGGG
RBOHE (At1g19230)	GTGATGCAAGATCAACCCTGA	GCCTTGCAAAATGTGTTCTCA
RBOHF (At1g64060)	GGTGTCATGAACGAAGTTGCA	AATGAGAGCAGAACGAGCATCA
CSD1 (At1g08830)	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
CSD2 (At2g28190)	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
FSD1 (At4g25100)	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
CAT1 (At1g20630)	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
APX1 (At1g07890)	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
APX2 (At1g07890)	GAGATGTGTTTGGTCGGATGG	CTCGAATCCTGAACGCTCC
GSH1 (At4g23100)	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
GSH2 (At5g27380)	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC
GST2 (At4g02520)	ATCACCAGTTCGACCCAGTG	CTCCTCTTCTGCAACAACGG
GST6 (At2g47730)	ATCTCCAGTTCGAGCTCATCC	CTCGAGAGCAGGAATTTGACC
GSTU26 (At1g17190)	CCCCAATCCTTCCCTCTGA	TGCCCATACCTTCCATGATG
MRP4 (At2g47800)	CCACCGGATTCCTACAGTGA	GCAAACAGAGACGGCCTCTC
OXI1 (At3g25250)	CGATTATTGTCCGGGACAGA	CTAATACAAGCTCCGCCGC
ANP1 (At1g09000)	AAGAGAGGACACTGCTCGTGG	TTGCGTCTGTTGCTCTTGAAG
ANP2 (At1g54960)	GGTGACTGGAAAAGCTCCTTG	TTGTCAGGGATTGGAGGATG
MEKK1 (At4g08500)	TGAGATATCGTGGCACAGCC	CCCGAAGCTGGTATCTTTGG
MKK2 (At4g29810)	GGATCCAAACAGTCGAAGCTC	TGCATCTGTGAAGTAGGACGC
MPK3 (At3g45640)	GACGTTTGACCCCAACAGAA	TGGCTTTTGACAGATTGGCTC
MPK4 (At4g01370)	ACATGTCGGCTGGTGCAGT	AATATGGGTGGCACAACGC
MPK6 (At2g43790)	TAAGTTCCCGACAGTGCATCC	GATGGGCCAATGCGTCTAA
CDPK1 (At1g18890)	CAAAGCTGGGCTTCAGAAGG	AAACCCATTTCCATCGACATC
SOS3 (At5g24270)	CGCTTCTTCACGAATCCGA	TCGTTTTTGCGGTCTGCTT
SOS2 (At5g35410)	ATTATCTTCGATCAAGGCCGG	GTTTCACCAGCAGCCTTTCTT
WRKY22 (At4g01250)	AAACCCATCAAAGGTTCACCA	GGGTCGGATCTATTTCGCTC
WRKY25 (At2g30250)	GAAAGATCCGCAGCAGACG	TCCCAATAATTTCACGAGCG
WRKY29 (At4g23550)	CATGGGCGTGGCGTAAATA	TTGTTTTCTTGCCAAACACCC
ZAT12 (At5g59820)	GTGCGAGTCACAAGAAGCCTAACA	GCGACGACGTTTTCACCTTCTTCA
GAPDH (At3g04120)	TGTCAGACTCGAGAAAGCTGC	CATCCTCGGTGTATCCAAGGA
GAPC (At1g13440)	TGGTGACAACAGGTCAAGCA	TGTAACCCCATTCGTTGTCG
ADH1 (At1g77120)	GAATCGCTGGTGCTTCTAGG	CTCAGCGATCACCTGTTGAA
LDH1 (At4g17260)	TGGTGGTGATGTTTTCCTCA	CTGCAGCTTCTCAGCCTCTT
PDC1 (At4g33070)	GGTGGTCCTAAGTTGCGTGT	CTGCTCCCCAATAAGTTCCA
PAL1 (At2g37040)	GCTTCATCCTTCTCGCTTCTG	ACGGGTACGTTGCGCTACA
PAL2 (At3g53260)	ACAGTTACGGAGTCACCACCG	CCGGCGTTCAAAAATCTAATG
ACT2 (AT3G18780)	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
At4g26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
At5g08290	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT

3.5.3 Hierarchical clustering of the gene expression data

Clustering analysis was performed to identify coordinately regulated genes (during a specific treatment), using the GenEx Software. The analysis was based on the "Ward's algoritm", an anova based clustering analysis. The distances are calculated based on the centroid linkage and expressed as an Euclidian distance measurement.

3.6 Analysis of enzymes and metabolites

3.6.1 Analysis of enzyme activities

Frozen leaf or root tissue (200 mg) was homogenized in 1 mL ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone. The homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 20,000*g* and 4 °C. The enzyme capacities, *i.e.* potential activity measured *in vitro* under non-limiting reaction conditions, were measured spectrophotometrically in the supernatant at 25 °C.

Guaiacol peroxidase and syringaldazine peroxidase capacities (GPX, SPX, EC 1.11.1.7) were measured at 436 nm and 530 nm according to Bergmeyer (Bergmeyer *et al.* 1974) and Imberty (Imberty *et al.* 1984), respectively. Ascorbate peroxidase capacity (APX, EC 1.11.1.11) was measured at 298 nm following the method of Gerbling (Gerbling *et al.* 1984). Analysis of superoxide dismutase (SOD, EC 1.15.1.1) capacity was based on the inhibition of cytochrome c (McCord and Fridovich 1969). Analysis of the capacities of glutathione reductase (GR, EC 1.6.4.2) and catalase (CAT, EC 1.11.1.6) were performed as described by Bergmeyer (1974).

3.6.2 Ascorbate and glutathione determination

AsA and GSSG were determined by HPLC analysis. Therefore 50 to 100 mg of tissue was ground in liquid nitrogen in a pre-cooled mortar. When a homogenous powder was obtained 400 µL of ice cold 6% (w/v) meta-phosphoric acid was added and the mixture was clarified by centrifugation at 16 000*g* at 4 °C for 10 minutes. The resulting supernatant was kept frozen or on ice until HPLC analysis. Antioxidants were separated on a 100 mm x 4.6 mm Polaris C18-A reversed phase HPLC column (3 µm particle size; 30°C; Varian) with an isocratic flow of 1 mL/min of the elution buffer (25 mM K/PO_4-buffer, pH 3.00). The components were quantified using a home made electrochemical detector with glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The purity and identity of the peaks were confirmed using a diode array detector (SPD-M10AVP, Shimadzu) which was placed on line with the electrochemical detector. The concentrations of oxidized DHA or GSH were measured indirectly as the difference between the total concentration of

antioxidants in a DTT-reduced fraction and the concentration in the sample prior to reduction. Reduction of the sample was obtained by incubation of an aliquot of the extract in 400 mM Tris and 200 mM DTT for 15 min in the dark. The pH of this mixture was checked to be between 6 and 7. After 15 min the pH was lowered again by 4-fold dilution in elution buffer prior to HPLC analysis.

3.7 Statistical analysis

The datasets were analyzed using linear mixed-effects models (Verbeke and Molenberghs, 2000). Transformations were applied when necessary to approximate normality. If there were enough data available, the covariance structure was chosen to take into account the heterogeneous variability for each different pot and/or treatment. This is a correction for the correlation between plants within one pot to guarantee that the observed statistical differences are only due to the treatment. The Dunnett multiple comparison adjustment was applied to obtain corrected *p*-values. The statistical analysis was performed using the MIXED procedure in SAS 9.1.

Chapter 4

Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress

Karen Smeets, Joske Ruytinx, Brahim Semane, Frank Van Belleghem, Tony Remans, Suzy Van Sanden, Jaco Vangronsveld, Ann Cuypers (2007). Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. Environmental and experimental botany. *In press*.

Abstract

The early antioxidative defence mechanisms were studied in *Arabidopsis thaliana* by applying a range of realistic Cd concentrations. Our data suggest that a 24h exposure to 20 µM $CdSO_4$ is already too toxic to study moderate toxicity, whereas a highly coordinated oxidative stress-related defence response could be observed after Cd application of 5 and 10 µM. Significant differences in transcript abundance of several genes involved in antioxidative defence were observed. The generation of superoxide seems the main cause of oxidative stress in the roots, whereas in the leaves hydrogen peroxide appears to be an important player. Furthermore, an increased transcript level of lipoxygenase, a potential inducer of oxidative stress, suggests a central role of this gene in causing the Cd-related redox imbalance. Our results show that Cd as a non-redox-active metal induces oxidative stress and indicate that the antioxidative defence system is moderated by the activation of different genes in different organs and cellular compartments.

4.1 Introduction

Since the past century, many regions worldwide suffer from heavy metal pollution due to anthropogenic activities. Mainly areas with high industrial or agricultural activities have to cope with increased levels of Cd in the soil. In these areas, Cd contamination is of growing concern because of its known toxicity to the environment and to human health (Vangronsveld and Clijsters 1994, Nawrot *et al.* 2006).

In plants, Cd causes severe physiological and morphological effects such as stunted growth, chlorosis and decreased reproducibility. At the cellular level, Cd interacts with biomolecules such as proteins and nucleic acids, is known to affect enzyme activities and causes alterations in membrane permeability (Sanita di Toppi and Gabbrielli 1999). Possible underlying mechanisms are the binding of Cd to sulfhydryl and/or carboxyl groups or the replacement of essential cofactors, e.g. Zn by Cd, which can lead to oxidative stress.

ROS (reactive oxygen species) are also produced under normal circumstances and plants have a well equipped antioxidative defence system to maintain the redox equilibrium. ROS are generated as by-products of some biochemical processes but also have important cellular functions such as signal transduction. They are involved in the regulation of several processes, such as mitosis, tropisms and cell death (Foyer and Noctor 2005a, Kangasjärvi *et al.* 2005). In non-stress conditions, ROS and other oxidants are balanced against the antioxidative defence system which is composed of enzymes as well as metabolites. Enzymes such as superoxide dismutases (SOD) are able to neutralize superoxide ($O_2^{\circ-}$), and catalases (CAT) and peroxidases (PX) have a role in quenching hydrogen peroxide (H_2O_2). All subcellular compartments contain specific antioxidative enzymes or metabolites which are coordinated and controlled by underlying regulatory mechanisms.

In stress situations, this redox equilibrium is disturbed and the increased ROS accumulation causes specific oxidative stress responses (Cuypers *et al.* 2001). Also Cd is able to provoke this disequilibrium. Previous research has shown that high Cd exposure as well as lower, more realistic, Cd concentrations are able to induce oxidative stress, which provokes an increase in metabolite content as

well as the activation of several antioxidative enzymes (Mench *et al.* 2003, Skorzynska-Polit *et al.* 2003, Smeets *et al.* 2005).

Since Cd itself is not redox-active, the increased ROS levels are likely to be induced via indirect mechanisms such as interaction with the antioxidative defence system, disruption of electron transport chains or induction of lipid peroxidation. The latter can be due to a Cd-mediated increase in lipoxygenase (LOX) activity. Lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids producing hydroperoxy fatty acids which can be subsequently metabolized via several secondary pathways to produce bioactive compounds such as jasmonate and oxylipins (Porta and Rocha-Sosa 2002). LOXes are involved in physiological processes such as growth and development, and are often induced during specific stress conditions (Ali *et al.* 2005a). According to previous research, the increase in LOX activity could be important in the oxidative stress induction after Cd application (Somashekaraiah *et al.* 1992). Another potential mechanism by which Cd can give rise to oxidative stress is via the production of H_2O_2 (as a result of superoxide dismutation or independent of this process) (Cho and Seo 2005). H_2O_2 can modulate the expression of various genes, such as antioxidative genes and modulators of the H_2O_2 production (Neill *et al.* 2002). In pea leaves, NADPH oxidase activity was shown to be the main source of H_2O_2 generation (via $O_2^{\circ-}$) after Cd exposure (Romero-Puertas *et al.* 2004, Maksymiec and Krupa 2005).

In summary, oxidative stress probably plays an important role in the cellular Cd toxicity. The aim of this study was to reveal the level of oxidative stress, its underlying mechanisms and their potential impact after moderate Cd exposure. In contrast to most of the previous research, Cd phytotoxicity was studied by applying low, realistic concentrations. A concentration range was determined based on concentrations found in the pore water of contaminated soils in North-Limburg (Belgium). Furthermore, whereas in previous research the antioxidative defence system was mostly analyzed at enzymatic level (enzyme activities), in this study several enzymes involved in the antioxidative defence and in the induction of oxidative stress were investigated at enzymatic as well as at transcriptional level. In order to improve our current knowledge concerning the spatial and temporal activation of the different oxidative stress-related effects after Cd exposure, both root and shoot samples were examined.

4.2 Results

4.2.1 Elemental profile and growth parameters

Three-week-old *Arabidopsis thaliana* seedlings were exposed to 0, 5, 10 and 20 µM $CdSO_4$ during 24h. A significant increase in root Cd content was observed as a function of the externally applied Cd concentration. In the leaves, a significant increase in Cd content was noticed after exposure to 5 and 10 µM $CdSO_4$. Exposure to 20 µM $CdSO_4$ resulted in a lower Cd content in the leaves than did the exposure to the lower Cd concentrations, but this was still significantly higher than in the unexposed seedlings. (figure 4.1)

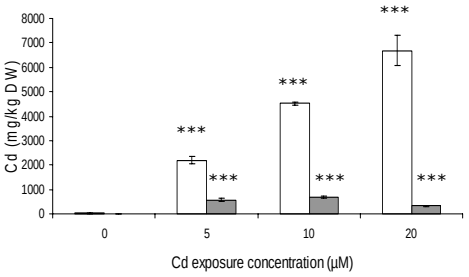


***Figure 4.1**: Cd content (mg kg^{-1} DW) in the roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0, 5, 10 or 20 µM $CdSO_4$ for 24h. Each point represents the mean of three biological replicates ± SE (significance levels: *: p<0.1; **: p<0.05; ***: p<0.01).*

Furthermore, significant differences were observed in the mineral nutrient content of plants exposed to 20 µM $CdSO_4$. The Ca and Cu contents of the roots were significantly increased compared to the control plants. In the leaves, significant decreases were observed in Mg, P, S, K, Ca and Mn contents after exposure of the plants to 20 µM $CdSO_4$. The leaf P content was also significantly decreased after exposure to the lower concentrations (table 4.1).

At higher Cd concentrations root fresh weight tended to decrease as compared to control seedlings, although the difference was not statistically significant. There was no apparent effect of Cd on leaf biomass (results not shown).

4.2.2 Gene expression

Transcript levels of several antioxidative enzymes were differentially altered as regards to the external Cd concentration. The transcript levels of *CSD2* (plastidic CuZnSOD) were decreased: application of 5 and 20 µM $CdSO_4$ resulted in significant decreases in both plant parts, whereas exposure to 10 µM $CdSO_4$ only caused significant differences in the leaves (figure 4.2A).

Table 4.1: *Elemental changes in Arabidopsis. thaliana under Cd exposure. Element concentration (mg kg^{-1} DW) of 3-week-old A. thaliana seedlings exposed to 0, 5, 10 or 20 µM $CdSO_4$. Values are mean ± SE of four independent biological replicates (significance levels: [(a)]: p-value<0.1;* [(b)]: p-value<0.05; [(c)]: p-value<0.01).

Element	Root 0µM Cd	Root 5µM Cd	Root 10µM Cd	Root 20µM Cd	Leaf 0µM Cd	Leaf 5µM Cd	Leaf 10µM Cd	Leaf 20µM Cd
Na	1466±167	2096±612	1955±488	1926±481	1495±214	1293±120	948±302	983±189
Mg	4199±731	2719±532	4497±849	4168±1909	9626±186	9171±230	9045±122	6558±84 [(c)]
P	11480±421	10554±360	11554±1011	12412±1489	10281±231	9511±60 [(b)]	9074±180 [(b)]	7086±142 [(b)]
S	5837±795	2719±532	4497±849	4167±1909	9407±260	9887±91	9540±370	6558±84 [(b)]
K	31824±1824	29079±2012	28281±5176	25483±5176	28121±687	28148±515	27397±346	20733±483 [(b)]
Ca	9864±1792	9949±2515	12906±3217	12374±4626 [(a)]	37351±695	33682±461	32998±889 [(a)]	25796±315 [(b)]
Mn	570±110	333±59	353±31	522±5	207±9	186±3	191±4	148±2 [(a)]
Fe	5097±512	4231±536	5661±991	6203±1374	594±121	433±286	479±137	174±13
Cu	21±2	40±3	34±1	53±9[(b)]	12±1	11±2	10±0.1	10±2
Zn	221±15	193±13	185±13	246±34	101±5	80±7	69±4	70±5

Additionally, both *FSD1* (plastidic FeSOD) and *MSD1* (mitochondrial MnSOD) transcript levels were significantly induced in the roots, whereas no significant differences were observed in the leaves (figure 4.2B+C). *FSD1* expression resulted in an increase up to 17 times the control value.

Transcript levels of *CAT1* (peroxisomal/mitochondrial CAT) appeared to be significantly increased by all Cd levels in the roots (figure 4.2D), but in shoots only when higher Cd levels were applied.

Furthermore, transcript levels of some important enzymes of the ascorbate-glutathione cycle were determined. For *APX1* (cytosolic APX) and *DHAR* (cytosolic DHAR), a bell-shaped expression pattern was observed in the leaves as a function of the Cd exposure concentration (figure 4.3A+B). Significant differences in *APX1* and *DHAR* levels were noticed after exposure to 10 µM $CdSO_4$. The other Cd concentrations also resulted in significant increases of leaf *APX1* transcript abundance. In the roots, a significant rise in *DHAR* transcript level was observed after an exposure to 10 and 20 µM $CdSO_4$ (figure 4.3B).

The *GR1* (cytosolic GR) gene was significantly increased in both roots and shoots for all Cd treatments (figure 4.3C). The relative mRNA level of *GPX2* (cytosolic GPX) was also measured and appeared to be increased in leaves for all Cd treatments, although this was only significant for 10 µM $CdSO_4$ (figure 4.3D).

Finally, the transcript level of *LOX1* was determined. A highly significant increase (6-10 times the control value) was observed in the roots after a 24h exposure to all concentrations. In contrast, no significant effects were observed in the leaves (figure 4.4).

A summary of all the transcriptional changes is presented in table 4.2.

Table 4.2: *Summary of the transcriptional changes*

Gene	Enzyme	Cellular compartment	Root	Leaf
CSD2	CuZnSOD	Plastid	↓	↓
FSD1	FeSOD	Plastid	↑	=
MSD1	MnSOD	Mitochondrion	↑	=
CAT1	CAT	Peroxisome	↑	↑
APX1	APX	Cytosol	=	↑
DHAR	DHAR	Cytosol	↑	↑
GR1	GR	Cytosol	↑	↑
GPX1	GPX	Cytosol	=	↑
LOX1	LOX	Unknown	↑	↑

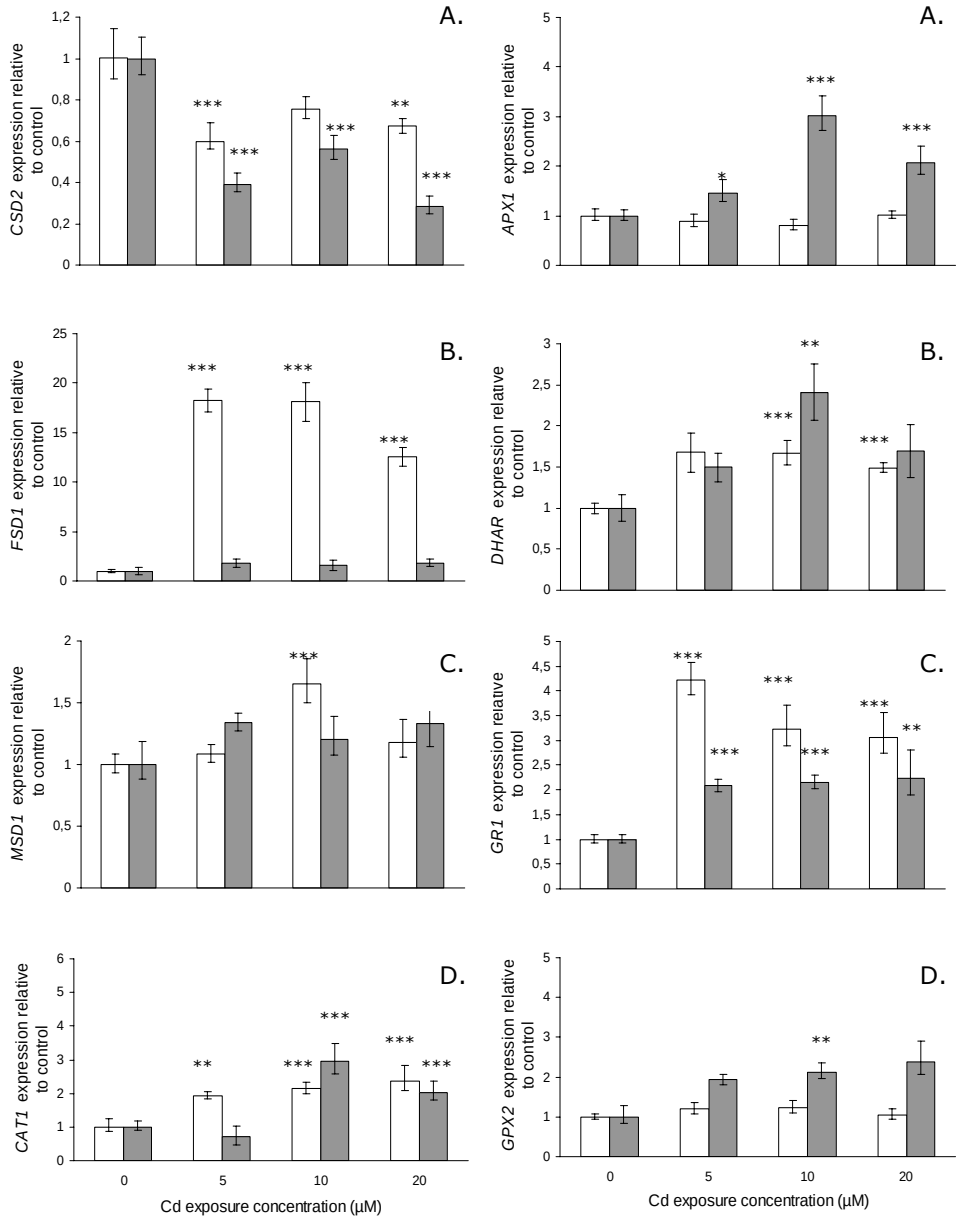


Figure 4.2 *: Transcript level of $O_2^{\circ-}$ - and H_2O_2 - scavenging enzymes, expressed relative to the control, in the roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0, 5, 10 or 20 µM $CdSO_4$ for 24h. Each point represents the mean of six biological replicates ± SE (significance levels: *: p<0.1; **: p<0.05; ***: p<0.01).*

Figure 4.3 *: Transcript level of genes related to the ascorbate-glutathione cycle, expressed relative to the control, in the roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0, 5, 10 or 20 µM $CdSO_4$ during 24h. Each point represents the mean of six biological replicates ± SE (significance levels: *: p<0.1; **: p<0.05; ***: p<0.01).*

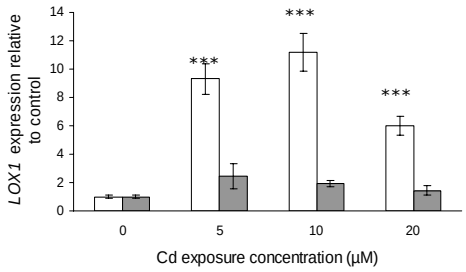


Figure 4.4: *Transcript level of lipoxygenase1, expressed relative to the control, in the roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0, 5, 10 or 20 µM $CdSO_4$ during 24h. Each point represents the mean of six biological replicates ± SE (significance levels: *:p<0.1; **: p<0.05; ***: p<0.01).*

4.2.3 Enzyme activities

Exposure to realistic Cd concentrations did not influence the total superoxide dismutase (SOD) activity nor did it change the catalase (CAT) activity (table 4.3) in the leaves of *Arabidopsis thaliana* seedlings. Compared to the untreated control, a numerical increase in CAT activity was observed, but these differences were not statistically significant. A decrease in glutathione reductase (GR) activity was observed for 5 and 10 µM Cd and it became significant after exposure to 20 µM $CdSO_4$.

In the leaves of *Arabidopsis thaliana* seedlings, exposure to increasing Cd concentrations resulted in a bell-shaped activity profile for some peroxidases (table 9.3). Compared to the untreated control, significant changes in guaiacol peroxidase (GPX), syringaldazine peroxidase (SPX) and ascorbate peroxidase (APX) activity were observed after exposure to 5 and 10 µM $CdSO_4$. However, exposure to 20 µM $CdSO_4$ resulted again in a slight decrease in the enzymatic activities but they were still significantly higher than the activities measured in the extracts of untreated plants.

Table 4.3: *Enzyme activities (mU g^{-1} FW) in the leaves of 3-week-old Arabidopsis thaliana seedlings exposed to 0, 5, 10 or 20 µM $CdSO_4$. Values are mean ± SE of eight independent biological replicates (significance levels: [(a)]: p-value<0.1; [(b)]: p-value<0.05; [(c)]: p-value<0.01).*

	SOD	CAT	GPX	SPX	APX	GR
0 µM	1062±90	560 ±62	794 ±81	2863±259	15523±2071	70±6
5 µM	935±173	712±59	1061 ±60 [(b)]	4019±262 [(b)]	19881±1086 [(a)]	50±6
10 µM	1083±88	695±38	1304 ±86 [(c)]	4725 ±298 [(c)]	21433±1233 [(b)]	52±6
20 µM	1005±137	668 ±60	1050 ±69 [(a)]	3784 ±464	17645±700	46±4[(a)]

4.3 Discussion

Cd is a non-redox-active metal but also induces oxidative stress (Shah *et al.* 2001, Romero-Puertas *et al.* 2004), even at low concentrations (Milone *et al.* 2003, Olmos *et al.* 2003, Smeets *et al.* 2005). The increased ROS levels are likely to be induced via indirect mechanisms such as interaction with the antioxidative defence system, disruption of electron transport chains or induction of lipid peroxidation. An increase in LOX activity has been implicated in the oxidative stress induction after Cd application (Somashekaraiah *et al.* 1992). In our hydroponic test system, 3-week-old *Arabidopsis* plants were exposed for 24h to environmentally realistic Cd concentrations. Increasing external concentrations of Cd led to increasing Cd contents in the roots (figure 4.1). This resulted in a strong increase in *LOX1* expression in the roots, whereas *LOX1* transcript levels in the leaves were not significantly affected (figure 4.4). Probably, lipid peroxidation increases at the site of exposure to Cd, *i.e.* in the roots, where it could be an important cause of the generation of ROS. Oxidative stress in the leaves could be generated via a different mechanism, either after transport of Cd into the leaves, or via oxidative stress signalling from root to shoot. Our results deliver some support for the hypothesis of oxidative stress signalling from root to shoot. Plants exposed to 5 and 10 µM $CdSO_4$ showed equal total Cd concentrations in the leaves, but differences in gene expression were measured for the antioxidative enzymes. This discrepancy can be due to the higher root Cd content in the plants exposed to 10 µM $CdSO_4$ and hence a different signalling in these plants. However, we cannot rule out the possibility that a different speciation and/or localisation of Cd in the leaves cause the differences in gene expression between 5 and 10 µM Cd.

At an exposure to 20 µM Cd, the Cd content in the leaves started to decrease again, but this was still significantly higher than in the untreated seedlings. In addition, an exposure to 20 µM $CdSO_4$ also led to significant decreases in the leaf content of mineral nutrients such as Mg, P, S, K, Ca and Mn. A possible explanation to these results could be that Cd affects xylem loading at higher concentrations, which suggests that a 24h exposure to 20 µM $CdSO_4$ is already highly toxic. This is also reflected in a relative decrease in root biomass when plants are exposed to 20 µM Cd. Cadmium content of the roots increased with higher external Cd concentrations, whereas for the nutrients, only a rise in Cu

and Ca was observed under the same conditions (table 4.1). The latter might be due to decreased transpiration.

Even before the morphological effects become visible, toxic internal Cd concentrations are interfering at the molecular and biochemical level (Lagriffoul *et al.* 1998). In order to gain more insight into the involvement of oxidative stress in response to Cd, we studied multiple components of the antioxidative defence that are localized in different cellular compartments, at both the transcriptional and enzymatic level. Some ROS, such as H_2O_2, can easily diffuse across the membranes, where they exert specific functions at the subcellular level (Corpas *et al.* 2001, Neill *et al.* 2002, Mittler *et al.* 2004). Hydrogen peroxide can be produced directly or as a result of $O_2^{\circ-}$ dismutation by SOD. In our study, changes in SOD expression were mainly found in the roots. Each treatment resulted in a significant increase (12-17 fold) in root *FSD1* (plastidic FeSOD) transcript level, whereas a small increase in *MSD1* (mitochondrial MnSOD) transcript level was only detected in the roots after exposure to 10 µM $CdSO_4$ (figure 4.2B+C). Both FSD1 and MSD1 can function as scavengers of superoxide radicals, and we suggest that this sharp increase in the expression reflects the high oxidative stress level in the roots caused by superoxide. A strong increase in the expression of plastidic FeSOD has also been reported in other stress situations, including metal stress (Okamoto *et al.* 2001, Ben Rejeb *et al.* 2004). The observed increases in transcript abundance might also compensate for the observed reduction in *CSD2* (plastidic CuZnSOD) transcript level (figure 4.2A). In the leaves, on the other hand, transcript levels of *CSD2*, *FSD1* and *MSD1*, as well as general SOD activity at the enzymatic level, are not increased (figure 4.2, table 4.3). This suggests that oxidative stress caused by Cd is not due to superoxide in the leaves.

In the leaves, we observed an increase in peroxidase activity, indicating that H_2O_2 is a major actor of oxidative stress in the leaves. Indeed, peroxidases are key components in the scavenging of H_2O_2, and GPX, SPX and APX enzymatic activities were increased in the leaves. Also transcript abundance of *APX1* and *GPX2* was upregulated in the leaves, whereas transcript levels in the roots remained unaffected (figure 4.3A+D). In the cytosol, enzymatic as well as metabolic components of the ascorbate-glutathione cycle are operating as H_2O_2-scavengers. Because of its regulating properties, cytosolic *APX1* has a central

role in the ROS gene network. Especially the antioxidative components of the chloroplasts are operating under the influence of *APX1* (Davletova *et al.* 2005). Also the transcript levels of DHAR, another cytosolic component of the ascorbate-glutathione cycle, increased in both leaves and roots after exposure to 10 µM $CdSO_4$ (figure 4.3B). Interestingly, the *GR1* gene was transcriptionally upregulated in both plant parts while a significant decrease of the GR enzyme activity was observed (figure 4.3C, table 4.3). It is possible that Cd interacts with the translation mechanisms, disturbs the activity of GR or even more likely influences the turnover of this enzyme. Indeed, an oxidative modification of this protein, which can result in a higher proteolytic degradation, has been reported after Cd exposure (Romero-Puertas *et al.* 2002). Other studies also suggested oxidative stress and protein denaturation as important components of Cd toxicity related responses (Suzuki *et al.* 2001).

Also catalases scavenge H_2O_2. Catalases are highly represented in the peroxisomes and play a central role in maintaining the cellular redox balance (Corpas *et al.* 2001, Orendi *et al.* 2001). In other stress situations such as wounding and heat, *CAT1* also responds at the transcript level (Guan and Scandalios 2000, Scandalios *et al.* 2000). We observed an increase in catalase transcription, but no significant change in catalase enzymatic activity in the leaves (figure 4.2D, table 4.3). These discrepancies can be due to the presence of multiple allo- or isozymes. Alternatively, Cd can cause an enhanced breakdown of the proteins, which in turn also leads to an enhanced transcription.

In conclusion, the cellular redox status is affected already 24h after the exposure of plants to environmentally realistic Cd concentrations. Exposure to 20 µM $CdSO_4$ seemed highly toxic, and the decreased relative root biomass and decreased ion content in the leaves lead us to believe that several physiological and cellular processes are strongly affected at this point. Previous studies (with higher exposure concentrations and/or longer exposure times) already showed the effect of Cd on the activity of several antioxidative enzymes. Our findings indicate that also a moderate Cd exposure triggers the antioxidative defence in different cellular compartments in the roots and shoots. Cd-related responses were examined at different levels to unravel some of the mechanisms behind these effects. Highly significant changes in the transcript level of several

antioxidative genes were found, but not all genes showed similar responses in the roots and leaves (table 4.2), suggesting that the underlying mechanism of oxidative stress is different in the roots and leaves. Our results indicate that the generation of superoxide and the lipoxygenase activity are the main causes of oxidative stress in the roots, whereas in the leaves H_2O_2 seems to be an important player. Whether this H_2O_2 is produced locally as a result of increased Cd content of the leaves, or whether it arrives as a signal from the roots, remains to be elucidated.

Chapter 5

Oxidative stress related mechanisms during cadmium and copper toxicity

Cuypers Ann, Smeets Karen, Ruytinx Joske, Horemans Nele, Vanhoudt Nathalie, Remans Tony, Opdenakker Kelly, Semane Brahim, Van Belleghem Frank, Guisez Yves, Colpaert Jan, Vangronsveld Jaco (2007). Oxidative stress as a modulator in Cd and Cu toxicity in *Arabidopsis thaliana* seedlings. *Submitted*.

Abstract

Production of reactive oxygen species (ROS) in metal exposed plants is often observed. To gain better insight in ROS generation and scavenging induced by a redox-active and non-active metal, *Arabidopsis thaliana*, cv. Columbia seedlings are exposed to sublethal concentrations of copper (Cu) and cadmium (Cd) for a 24h period. Responses to both metals are investigated at the level of element composition, lipid peroxidation, H_2O_2 production, cellular redox state, gene expression and activities of ROS producing and antioxidative enzymes.

In roots, metal exposure results in high stress intensity presumably 'sensed' by the plasma membrane leading to strong increases in lipid peroxidation and a high induction of the cytoplasmic lipoxygenase gene. Due to its redox properties Cu directly induces an oxidative burst, whereas ROS produced by NADPH oxidases play an important role in case of Cd stres. In leaves, metal concentrations are much lower; Cu retention in roots is nearly complete and interorgan signalling is hypothesized to be involved in responses to plant Cu exposure. Because of lower metal concentrations in leaves, the direct metal stress intensity is supposed to be mild. Plasma membranes and chloroplasts are suggested to be the 'sensing sites'. Obviously, the interplay between production and scavenging of ROS is balanced, and a major emphasis is attributed to the role of the cellular redox state in intracellular signalling. Our results suggest that plant responses differ in roots and shoots depending on the metal stress intensity and that different intracellular 'sensing and signalling' mechanisms are involved in Cd and Cu toxicity.

5.1 Introduction

Copper is an essential redox-active micronutrient that is involved in many physiological processes in plants and hence is required for normal growth and development (Yruela 2005). The redox properties that make Cu an essential element, also contribute to its inherent toxicity. Excess Cu can disturb normal development by adversely affecting biochemical reactions and physiological processes in plants. Cadmium is a non-essential element that negatively affects plant growth and development. Cadmium is toxic presumably because it can replace some essential elements that play a key role in active sites of enzymes and due to its high affinity for sulfhydryl groups (Benavides *et al.* 2005).

Decreased biomass production has commonly been observed in plants subjected to Cd or Cu stress; nevertheless it is difficult to detect a common (path)way of action at the cellular level, due to complex interactions between metal ions and metabolism. Exposure to either metal results in cellular damage generated by reactive oxygen species (ROS) (Chaoui and El Ferjani 2005, Semane *et al.* 2007). Because of the toxic effects of ROS, it is key to keep their production and detoxification under tight control. Mittler *et al.* (2004) describe a large gene network consisting of at least 152 genes in *Arabidopsis* controlling the delicate balance between ROS toxicity and ROS signalling.

Despite our knowledge regarding toxic responses towards heavy metals and detoxification mechanisms, information on regulation and signal transduction is rather limited. Kacperska (2004) describes possible ways in signal sensing engaged in plant responses to various abiotic stresses, suggesting a complex network depending on the intensity of the stressor. Multiple studies emphasize the existence of extensive cross-talk between different signal transduction pathways in plant cells (Knight and Knight 2001, Mithofer *et al.* 2004, Bright *et al.* 2006). Because hydrogen peroxide (H_2O_2) production is an immediate response to increased Cd or Cu, it is probably a key molecule that can trigger signal transduction events after plant metal exposure (Mithofer *et al.* 2004, Bhattacharjee 2005, Maksymiec 2007). To unravel the complexity of "common" and/or "specific" signals involved in heavy metal stress responses, knowledge of the production and scavenging of ROS under Cd or Cu exposure is essential.

When toxic metals are taken up from soil solution into root cells, the plasma membranes of these cells can be considered as a primary target for metal action (Vangronsveld and Clijsters 1994). Membrane lipid peroxidation is a very sensitive response caused by metal stress and is initiated by a number of ROS or by the enzyme lipoxygenase (Cuypers *et al.* 2000, Shah *et al.* 2001, Mithofer *et al.* 2004, Smeets *et al.* 2005, Skorzynska-Polit *et al.* 2006). Recent findings propose an important role for NADPH oxidases in ROS production and signal transduction under abiotic stress (Jiang and Zhang 2003, Torres and Dangl 2005, Hao *et al.* 2006). NADPH oxidase transfers electrons from cytoplasmic NADPH to O_2 to form superoxide radicals ($O_2^{\circ-}$), followed by its dismutation to H_2O_2. Furthermore organelles with a highly oxidizing metabolic rate or possessing electron transport chains, such as chloroplasts, mitochondria and peroxisomes, are major sources of ROS production in plant cells (Mittler *et al.* 2004).

Plants possess an efficient antioxidant defence system consisting of enzymes and non-enzymatic compounds, such as ascorbate (AsA) and glutathione (GSH). Sequential and contemporaneous action of these antioxidative metabolites with catalases (CAT), superoxide dismutases (SOD) and peroxidases (PXs) makes up the cells antioxidative power, that maintains the cellular redox homeostasis within certain limits (Mittler *et al.* 2004). Furthermore, the ascorbate-glutathione cycle plays a central role in the antioxidant defence mechanism in plant cells (Foyer and Noctor 2003, Foyer and Noctor 2005a, Foyer and Noctor 2005b).

A number of authors have demonstrated responses at the biochemical-physiological level and on signal transduction pathways induced by exposure to relatively high (acute) heavy metal concentrations in plants (Jonak *et al.* 2004, Maksymiec and Krupa 2005, Yeh *et al.* 2007). Here, we aim to identify and to compare the interactions of Cu, a redox-active micronutrient, and Cd, a non-redox-active, non-essential element, on the cellular redox balance. *Arabidopsis thaliana* plants were exposed during 24h to low metal concentrations (5 and 10 µM Cd or 2 and 5 µM Cu), comparable to those commonly found in pore water of contaminated soils. We identified ROS mediated responses that can be linked to signal transduction pathways as they might occur under environmentally realistic stress conditions

5.2 Results

5.2.1 Altered nutrient acquisition and membrane integrity in Arabidopsis thaliana seedlings under Cd/Cu stress

To get an indication of the integrity of cell membranes, analysis of some macro- and micronutrients were performed (table 5.1). A differential treatment response was found for the K-content of roots: Cd exposure did not affect the K-content, whereas Cu toxicity resulted in a significant decrease in K-concentration suggesting severe K-leakage from the root cells. Membrane damage could also be deduced from the analysis of lipid peroxidation products using a thiobarbituric acid determination (figure 5.1). Exposure to Cu increases lipid peroxidation in roots more strongly than Cd treatment. In leaves however, no significant differences in lipid peroxidation were found under both treatments. Metal transport to the aerial parts was limited with a root to shoot transfer of 5-6 for Cd and nearly complete retention of Cu in the roots. This coincides with a lower K-decrease in leaves of Cu-exposed plants (table 5.1), but it has to be noticed that the Fe concentration in leaves rose significantly after exposure to 5 µM Cu.

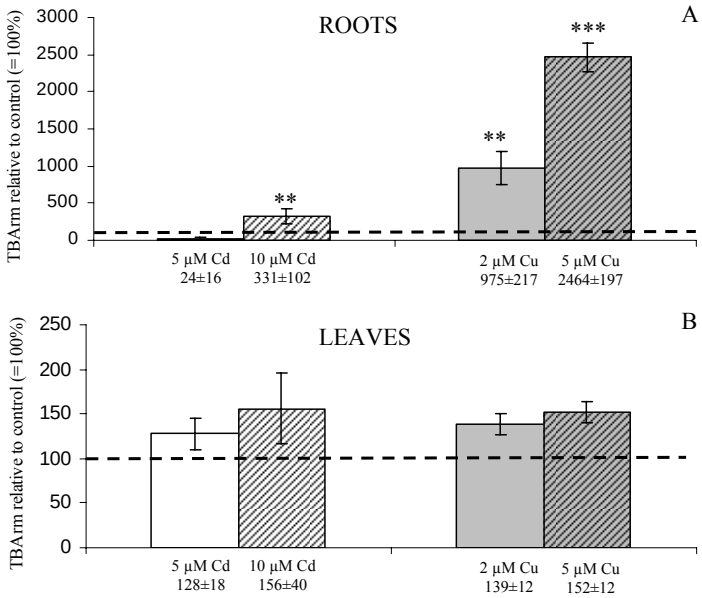


Figure 5.1: *Lipid peroxidation measurement was based on the amount of TBA reactive metabolites (TBAm) and is expressed relative to the control (100%: dashed line) in roots (A) and leaves (B) of 3-week-old seedlings exposed to 5 µM Cd (white, open) – 10 µM Cd (white, striped) or 2 µM Cu (grey, open) – 5 µM Cu (grey, striped) over a 24h period or grown under control conditions. Values are mean ± S.E. of at least 6 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

Table 5.1: *Element concentration (mg kg^{-1} DW) in roots and leaves of 3-week-old seedlings exposed to 0, 5, 10µM Cd or 0, 2, 5µM Cu during 24h. Values are mean ± S.E. of at least 3 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1). ND: not detectable.*

Element	Roots-Cadmium			Leaves-Cadmium		
	Control	5 µM Cd	10 µM Cd	Control	5 µM Cd	10 µM Cd
Mg	4199 ± 731	2719 ± 532	4497 ± 849	9626 ± 186	9171 ± 230	9045 ± 122
K	31824 ± 1824	29079 ± 2012	28281 ± 5176	28121 ± 687	28148 ± 515	27397 ± 346
Ca	9864 ± 1792	9949 ± 2515	12906 ± 3217	37351 ± 695	33682 ± 461	32998 ± 889*
Mn	570 ± 110	333 ± 59	353 ± 31	207 ± 9	186 ± 3	191 ± 4
Fe	5097 ± 512	4231 ± 536	5661 ± 991	245 ± 50	179 ± 118	198 ± 57
Cu	21 ± 2	40 ± 3	34 ± 1	12 ± 1	11 ± 2	10 ± 0.1
Zn	221 ± 15	193 ± 13	185 ± 13	101 ± 5	80 ± 7	69 ± 4
Cd	ND	3102 ± 1382***	4630 ± 697***	ND	654 ± 6***	764 ± 5***

Element	Roots-Copper			Leaves-Copper		
	Control	2 µM Cu	5 µM Cu	Control	2 µM Cu	5 µM Cu
Mg	8370 ± 263	8869 ± 310	7399 ± 352*	7311 ± 62	6197 ± 95***	6508 ± 335
K	46623 ± 2644	8049 ± 607***	3145 ± 375***	25609 ± 599	21589 ± 1199*	20806 ± 583***
Ca	14935 ± 481	19767 ± 653***	17509 ± 759*	34065 ± 323	288559 ± 719***	30035 ± 1127*
Mn	403 ± 56	275 ± 11	199 ± 25*	158 ± 1	145 ± 4*	148 ± 7
Fe	7821 ± 282	10080 ± 453	9097 ± 558	128 ± 19	130 ± 23	406 ± 187**
Cu	15 ± 0.1	1464 ± 44***	3307 ± 168***	5.0 ± 0.2	8.0 ± 0.8**	8.0 ± 0.3***
Zn	110 ± 9	182 ± 49	98 ± 2	38 ± 1	38 ± 2	32 ± 1**

5.2.2 The cellular redox state in Arabidopsis is affected after application of Cd or Cu

The content of both metabolites was determined in *Arabidopsis* roots and shoots after a 24h exposure to different Cd and Cu concentrations (table 5.2).

In roots, a differential response was observed between Cd-exposed and Cu-exposed plants. Whereas a strong depletion of GSH was noticed in roots after 5 µM Cu application, the total GSH amount in Cd-exposed roots remained similar to the controls. Nevertheless, the glutathione redox state (GSSG/GSH) was

increased under both stress conditions, but to a greater extent in Cu-exposed plants. Also for AsA contents a strong decrease in 5 µM Cu-exposed plants was noticed, furthermore the ascorbate pool was almost completely oxidized under these circumstances. Exposure to Cd resulted in a slight increase of total AsA, but without alterations in the redox state (DHA/AsA).

In leaves, significant differences were only observed in plants exposed to 5 µM Cu.

5.2.3 Possible sources of ROS production under Cd/Cu stress

The H_2O_2 concentration was analyzed in roots and shoots of *Arabidopsis thaliana* seedlings exposed to different concentrations of Cd and Cu (figure 5.2). A strong elevation of the H_2O_2 content was observed in both roots and leaves of Cd-exposed plants, but only in leaves of 5 µM Cu-exposed plants.

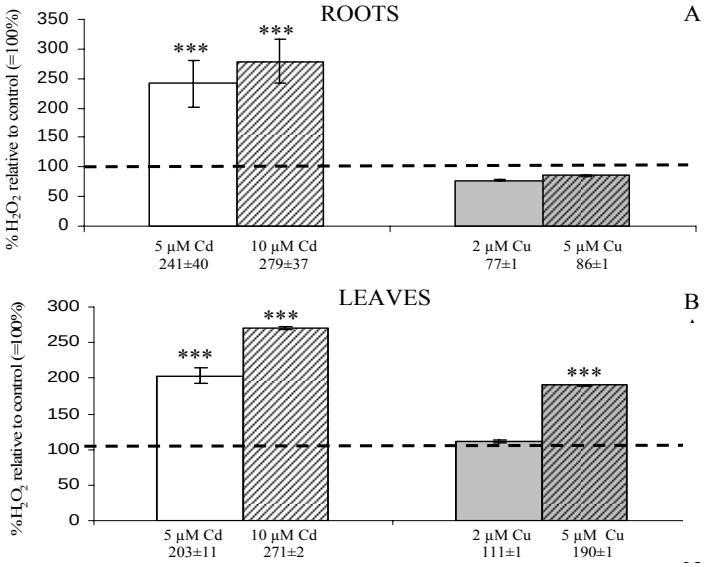


Figure 5.2: *H_2O_2 production is expressed relative to the control (100%: dashed line) in roots (A) and leaves (B) of 3-week-old seedlings exposed to 5 µM Cd (white, open) – 10 µM Cd (white, striped) or 2 µM Cu (grey, open) – 5 µM Cu (grey, striped) over a 24h period or grown under control conditions. Values are mean ± S.E. of at least 6 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

Gene expression, using real time PCR, was performed to investigate the implication of lipoxygenases (LOX), both cytoplasmic and plastidic isoforms, and NADPH oxidases in ROS-mediated cellular responses after exposure of *Arabidopsis* seedlings to Cd or Cu (figure 5.3). In roots, the cytosolic *LOX1* was strongly induced under both metal stresses, whereas *LOX2* gene expression was reduced in Cu-exposed plants and after exposure to 5 µM Cd. Both LOX mRNAs

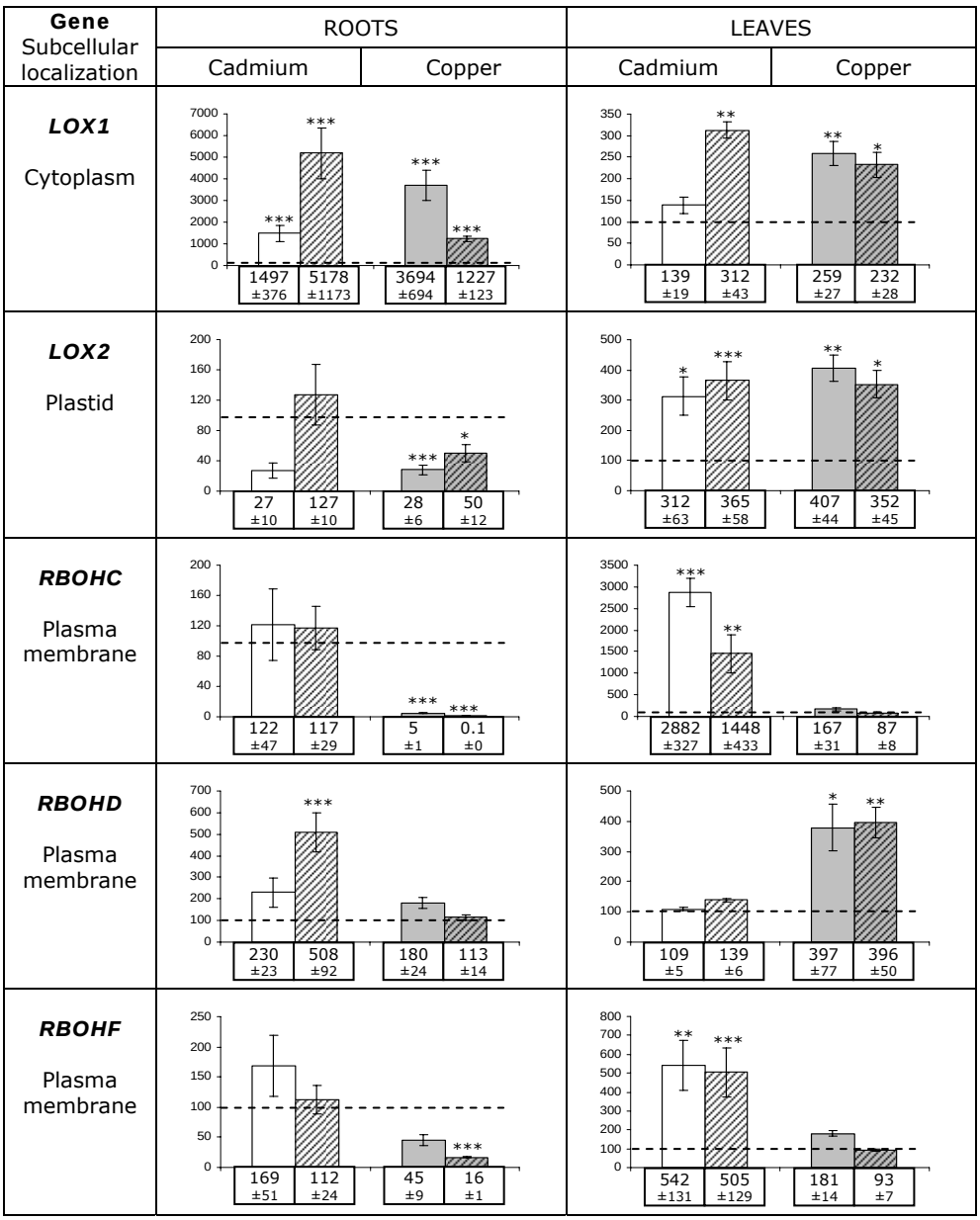


Figure 5.3: *Alteration in gene expression of ROS producing enzymes after exposure to Cd or Cu. Gene expression of ROS producing enzymes relatively expressed to the control (100%: dashed line). Analyses were performed in roots and leaves of 3-week-old Arabidopsis seedlings exposed to 5 µM Cd (white, open) – 10 µM Cd (white, striped) or 2 µM Cu (grey, open) – 5 µM Cu (grey, striped) over a 24h period or grown under control conditions. Information of the genes (lipoxygenases: LOX1, LOX2; NADPH oxidases: RBOHC, RBOHD, RBOHF) and their subcellular localization are given ahead of the complementary results, in which the first column represents results of roots and in the second column the results of leaves are shown. Values are mean ± S.E. of at least 8 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1)*

were enhanced in the leaves under both treatments, but with emphasis on *LOX2*.

Table 5.2: *Metabolite concentrations (relative to the control of leaf = 100%) in roots and leaves of 3-week-old seedlings exposed to 5 and 10µM Cd or 2 and 5µM Cu during 24h. Total metabolite content represents both the reduced and oxidized form. The redox state is the ratio between the reduced form of the metabolite and its oxidized form (AsA: reduced ascorbate, DHA: dehydroascorbate; GSH: reduced glutathione, GSSG: glutathione disulfide). Values are mean ± S.E. of at least 3 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

METABOLITES	ROOTS-CADMIUM			LEAVES-CADMIUM		
	Control	5 µM Cd	10 µM Cd	Control	5 µM Cd	10 µM Cd
(AsA + DHA)	17.9 ± 2.4	26.4 ± 2.1*	21.9 ± 1.2	100.0 ± 4.4	106.5 ± 7.4	99.0 ± 6.0
AsA	13.1 ± 2.4	19.7 ± 1.7	18.2 ± 1.5	83.9 ± 2.0	75.7 ± 11.8	81.2 ± 1.2
DHA	4.8 ± 0.9	6.6 ± 0.4	3.6 ± 1.3	16.1 ± 3.0	30.8 ± 8.4	17.7 ± 4.7
DHA/AsA	0.39 ± 0.10	0.34 ± 0.01	0.22 ± 0.10	0.19 ± 0.07	0.45 ± 0.15	0.21 ± 0.05
(GSH + GSSG)	25.8 ± 1.0	38.1 ± 2.6	24.7 ± 2.2	100.0 ± 2.3	123.5 ± 3.6	132.5 ± 15.3
GSH	24.9 ± 0.8	32.7 ± 4.4	20.7 ± 1.7	90.5 ± 2.7	97.1 ± 6.9	89.6 ± 2.7
GSSG	0.83 ± 0.20	5.36 ± 1.9	3.9 ± 1.6	9.5 ± 1.2	26.4 ± 3.7	42.9 ± 16.7
GSSG/GSH	0.033 ± 0.006	0.19 ± 0.09*	0.19 ± 0.08*	0.11 ± 0.016	0.28 ± 0.05	0.49 ± 0.20

METABOLITES	ROOTS-COPPER			LEAVES-COPPER		
	Control	2 µM Cu	5 µM Cu	Control	2 µM Cu	5 µM Cu
(AsA + DHA)	17.9 ± 2.4	10.5 ± 0.9*	2.7 ± 0.3**	100.0 ± 4.4	111.1 ± 9.1	141.2 ± 4.9***
AsA	13.1 ± 2.4	3.9 ± 0.4***	0.6 ± 0.3***	83.9 ± 2.0	91.4 ± 11.0	108.0 ± 11.8
DHA	4.8 ± 0.9	6.5 ± 0.5	2.1 ± 0.2*	16.1 ± 3.0	19.7 ± 3.2	33.2 ± 8.9
DHA/AsA	0.39 ± 0.10	1.67 ± 0.06**	2.28 ± 0.40***	0.19 ± 0.07	0.23 ± 0.05	0.33 ± 0.12
(GSH + GSSG)	25.8 ± 1.0	37.3 ± 7.3	5.4 ± 0.6*	100.0 ± 2.3	107.9 ± 9.4	155.5 ± 5.0*
GSH	24.9 ± 0.8	22.7 ± 1.7	2.8 ± 0.7*	90.5 ± 2.7	89.3 ± 10.6	135.3 ± 8.3**
GSSG	0.83 ± 0.20	14.6 ± 5.6	2.6 ± 0.3**	9.5 ± 1.2	18.6 ± 1.9	20.2 ± 3.3*
GSSG/GSH	0.033 ± 0.006	0.64 ± 0.08***	1.16 ± 0.49***	0.11 ± 0.016	0.22 ± 0.04	0.15 ± 0.03*

An important source of ROS production at the plasma membrane is the $O_2^{\circ-}$ production by NADPH-oxidases, a process that is strongly affected by the metal supplied. The analyses demonstrated that after Cu treatment in roots there was an inhibition of the gene expression of NADPH oxidases (*RBOH-C*/F). Cadmium treatment on the other hand resulted in a significant induction of *RBOHD* gene expression. In leaves, the highest induction caused by Cd exposure was found for the *RBOHC* gene, but also a strong induction of the *RBOHF* gene was noticed after Cd treatment. *RBOHD* was significantly induced after Cu exposure.

5.2.4 Enzymatic ROS scavenging under Cd/Cu stress

A reduction in gene expression of all superoxide scavenging enzymes was noticed in roots of Cu-exposed plants (figure 5.4). Looking at responses to Cd stress, an induction of *FSD1* gene expression was most obvious in the different SOD-isoforms, but also an increase in *MSD1* gene expression (10 µM Cd) was noticed. *CSD1* was reduced in roots after exposure to 5 µM Cd. Concerning enzymes involved in scavenging of H_2O_2, a strong induction occurred for *APX2* for all treatments in contrast to a reduction in *APX1* gene expression under Cu stress (figure 5.5). *CAT1* transcripts were enhanced after exposure to 10 µM Cd or 2 µM Cu. Significant difference was also found for the gene expression of the GSH recycling enzyme glutathione reductase (GR) which was induced in Cd-exposed roots but reduced after Cu treatment.

In leaves, an induction of the SOD gene expression was mainly restricted to *CSD1* under Cu stress, with a strong reduction of *FSD1*. After Cd application, no significant differences were noticed in the gene expression of the SOD isoforms studied, except a reduction of *FSD1* after 5 µM Cd exposure. In contrast, an induction of the H_2O_2 scavenging enzyme *APX1* was observed under both treatments. Enhancement of the *CAT1* and *GR1* gene expression in leaves was restricted to Cd treatment.

Apart from total SOD activities that were not affected by Cd nor by Cu in roots, the antioxidative capacities were significantly diminished in Cu-exposed roots, but slightly increased under Cd stress (table 5.3). Similar observations were made for leaves, in which SOD activities were not significantly altered, and the activities of enzymes coping with H_2O_2 were significantly elevated after Cd

application. Significant increase in CAT and GR activity was also noticed in leaves of Cu-exposed plants.

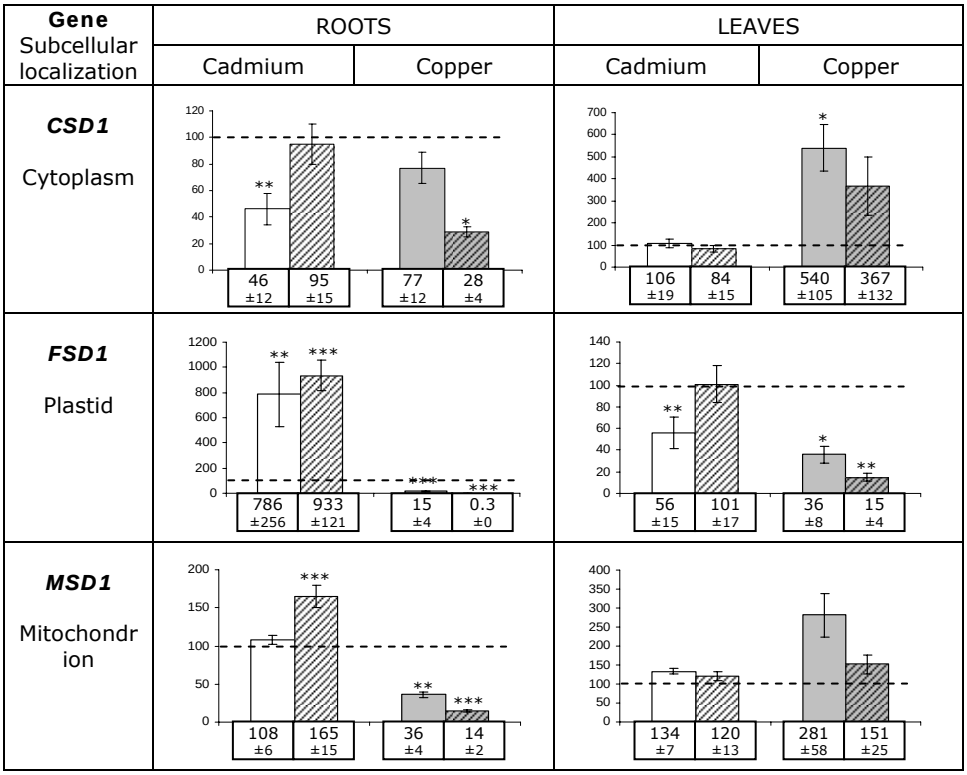


Figure 5.4: *Differences in the gene expression of $O_2^{\bullet-}$ scavenging enzymes after Cd or Cu application. Gene expression of $O_2^{\bullet-}$ scavenging enzymes (superoxide dismutases: SOD) relatively expressed to the control (100%: dashed line). Analysis were performed in roots and leaves of 3-week-old Arabidopsis seedlings exposed to 5 µM Cd (white, open) – 10 µM Cd (white, striped) or 2 µM Cu (grey, open) – 5 µM Cu (grey, striped) over a 24h period or grown under control conditions. Information of the genes (CuZnSOD: CSD1; FeSOD: FSD1; MnSOD: MSD1) and their subcellular localization are given ahead of the complementary results, in which the first column represents results of roots and in the second column the results of leaves are shown. Values are mean ± S.E. of at least 8 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

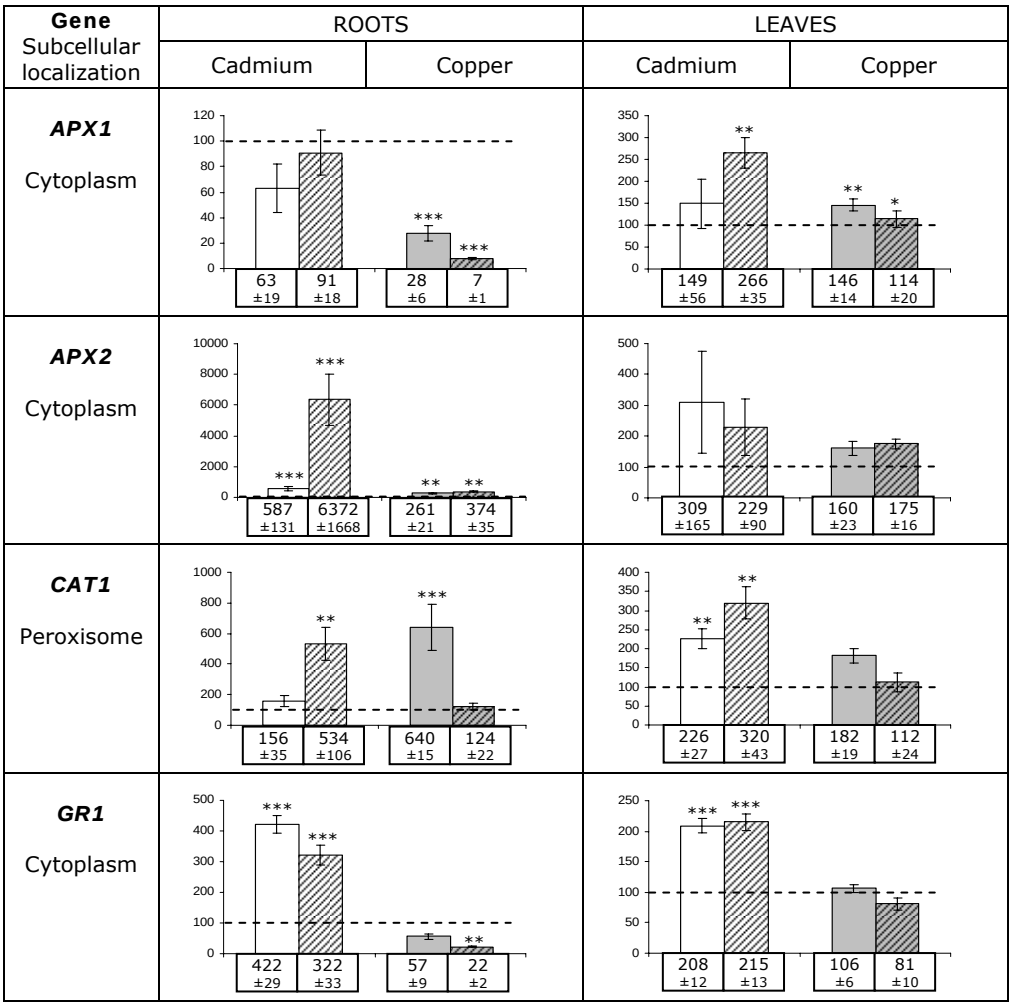


Figure 5.5: *Gene expression of H_2O_2-scavenging enzymes and GSH-related enzymes under both Cd and Cu stress. Gene expression of H_2O_2 scavenging enzymes and glutathione reductase (GR) relatively expressed to the control (100%: dashed line). Analysis were performed in roots and leaves of 3-week-old Arabidopsis seedlings exposed to 5 µM Cd (white, open) – 10 µM Cd (white, striped) or 2 µM Cu (grey, open) – 5 µM Cu (grey, striped) over a 24h period or grown under control conditions. Information of the genes (APX: APX1-2; CAT: CAT1; GR: GR1) and their subcellular localization are given ahead of the complementary results, in which the first column represents results of roots and in the second column the results of leaves are shown. Values are mean ± S.E. of at least 8 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

***Table 5.3**: Activities of antioxidative enzymes in roots and leaves of 3-week-old seedlings exposed to 0, 5, 10 µM Cd or 0, 2, 5 µM Cu over a 24h period or grown under control conditions. Enzyme activities were measured on crude root and leaf extracts [SOD: superoxide dismutase ($Ug^{-1}FW$); CAT: catalase ($mUg^{-1}FW$); APX: ascorbate peroxidase ($mUg^{-1}FW$); GR: glutathione reductase ($mUg^{-1}FW$)]. Values are mean ± S.E. of at least 4 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

Enzyme Activities	Roots-Cadmium			Leaves-Cadmium		
	Control	5 µM Cd	10 µM Cd	Control	5 µM Cd	10 µM Cd
SOD	1309 ± 157	1313 ± 189	1268 ± 102	1062 ± 90	935 ± 173	1083 ± 88
CAT	123 ± 26	167 ± 10*	132 ± 25	560 ± 62	712 ± 59	695 ± 38
APX	6107 ± 1016	8776 ± 1134	7867 ± 812	15523 ± 2071	19881 ± 1086*	21433 ± 1233**
GR	19 ± 3	36 ± 6*	17 ± 0.7	70 ± 6	50 ± 6	52 ± 6

Enzyme Activities	Roots-Copper			Leaves-Copper		
	Control	2 µM Cu	5 µM Cu	Control	2 µM Cu	5 µM Cu
SOD	1658 ± 163	1808 ± 82	1808 ± 136	743 ± 201	830 ± 137	1021 ± 163
CAT	76 ± 10	53 ± 4	31 ± 5**	121 ± 20	237 ± 82	244 ± 21**
APX	5883 ± 395	2384 ± 263***	3174 ± 350***	12867 ± 2701	19423 ± 3760	16701 ± 2030
GR	24 ± 3	25 ± 1	18 ± 2	76 ± 4	89 ± 5	96 ± 2**

5.3 Discussion

Elevated metal concentrations in the environment cause great losses in crop production world-wide. Although the overall response is similar, it is important to better understand the underlying molecular mechanisms. This information is most useful to develop or adjust strategies for growing non-food crops on heavy metal-contaminated agricultural soils, whether or not aiming phytoremediation. In the present study, we have analyzed different routes of ROS production and ROS scavenging under Cd and Cu stress in *Arabidopsis* seedlings. 5 and 10 µM Cd or 2 and 5 µM Cu concentrations were supplied to the roots of 3-week-old *Arabidopsis* seedlings and different responses at the molecular and biochemical level were investigated after 24h.

The results show a high Cu or Cd content in roots, whereas a much lower content was observed in leaves (table 5.1). Because of the different chemical

properties of Cu and Cd, differences in signal transduction pathways can be expected. In figure 5.6, a hypothesis for possible routes of ROS mediated-signalling under metal stress is developed, based on the results obtained in the present study.

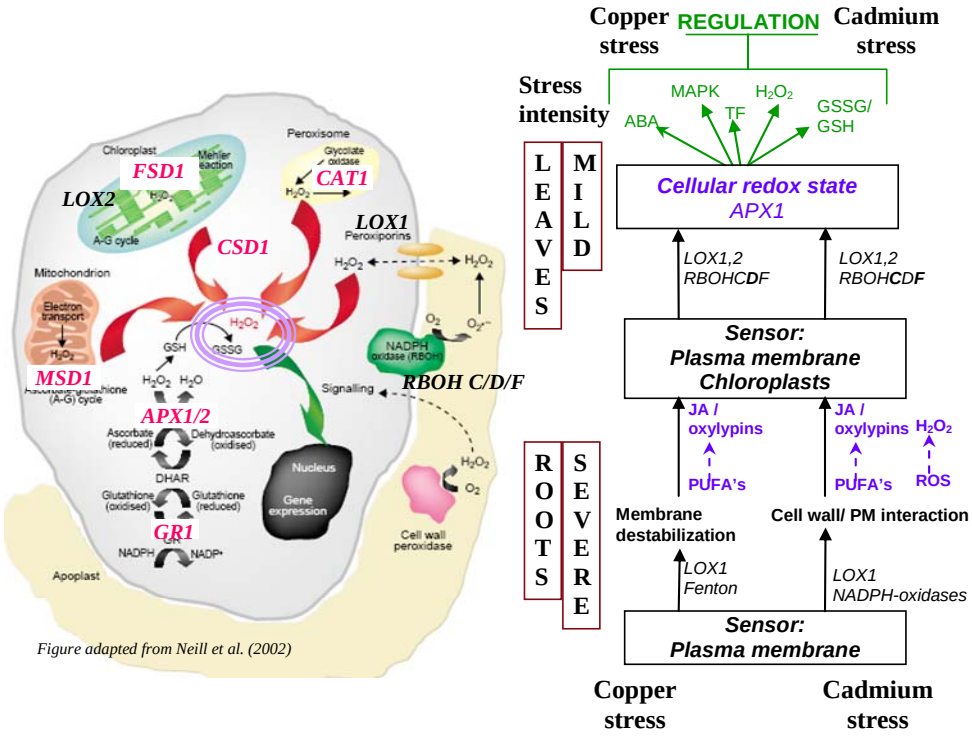


Figure adapted from Neill et al. (2002)

Figure 5.6: *Possible signal transduction routes under Cd and Cu stress in roots and leaves of 3-week-old Arabidopsis seedlings exposed to different Cd and Cu concentrations over a 24h period (Figure adapted from Neill et al., 2002). Stress intensities and hence stress sensing is different in both plant organs. Roots are exposed to severe, direct stress and membranes are the first target. Due to high LOX gene expression oxylipins and jasmonic acid (JA) could be proposed as molecules for long distance signalling under both conditions. Cd stress, which is much less severe than Cu under the conditions studied, induces high gene expression of NADPH oxidases, indicating that here also ROS might be involved in signalling from roots to shoots. At the leaf level the stress intensity is mild and therefore both the plasma membrane and the chloroplast seem to be sensors of metal stress. Due to the important induction of NADPH oxidases gene expression ROS as well as ABA can be intracellular signalling molecules under Cd or Cu stress. Furthermore the cellular redox state is important and probably is involved in subsequent regulation under both stress conditions. In literature MAPKinases have been shown to be important in Cd and Cu*

5.3.1 Oxidative stress as a modulator in Cd and Cu stress in roots

As soon as root cells come into contact with a nutrient solution containing toxic concentrations of heavy metals, their plasma membranes are subjected to oxidative damage. The early burst in ROS activity is most likely the result of the elevated NADPH-oxidase dependent $O_2°^-$ production, the activity of cell wall peroxidases and lipoxygenases, rather than a suppression of ROS scavenging mechanisms which more likely become involved in a later phase. Obviously the Cd and Cu concentrations supplied had a different impact on membrane integrity of root cells. It appeared that Cu was much more cytotoxic to the root cells, with a major increase in lipid peroxidation (figure 5.1) and a striking decrease of the K-content (table 5.1). ROS-mediated lipid peroxidation occurs under biotic and abiotic stresses (Maksymiec and Krupa 2005, Montillet *et al.* 2005). Redox-active metals, such as Cu, can enhance the production of hydroxylradicals (Fenton reaction) and further stimulate lipid peroxidation. Reduction of the oxidized metal ion can be achieved by the Haber-Weiss reaction with $O_2°^-$ as a substrate. Lipid peroxidation initiated by cytoplasmic lipoxygenases seems to play a prominent role under both metal exposures in our study (figure 5.3). Alternatively, products of LOX activities and subsequent reactions can give rise to a diverse array of structurally different oxylipins (Mithofer *et al.* 2004). Many oxylipins, in particular those belonging to the jasmonate (JA) family, are discussed as general inter- and intracellular signalling compounds involved in multiple defence reactions. In *Arabidopsis* seedlings, used in the present investigation, the maximum expression of *LOX1*, located in the cytoplasm, is found in roots (figure 5.3); *LOX1* has been shown to be induced by methyl jasmonate (MeJA) (Skorzynska-Polit *et al.* 2006). Jasmonates (JA) are involved in severe stressors giving rise to alarm responses (Kacperska 2004) and are possibly important signalling routes under Cd and Cu stress (Maksymiec and Krupa 2005, Kumari *et al.* 2006, Maksymiec 2007).

Whereas H_2O_2 production is clearly involved in the Cd responses (figure 5.2), it is plausible that produced H_2O_2 in Cu-exposed plants is immediately converted to other ROS. Multiple studies have demonstrated the involvement of NADPH oxidases in ROS production under abiotic and biotic stresses (Torres and Dangl 2005, Hao *et al.* 2006, Sagi and Fluhr 2006). A reduction of NADPH oxidases gene expression was observed in Cu-exposed roots, whereas an induction of

NADPH oxidases (*RBOH D*) was observed under Cd stress (figure 5.3) confirming their importance in a ROS-mediated Cd response in roots.

In our study the antioxidative protection, essential to counterbalance a high ROS production, was restricted to *CAT1* and *APX2* gene expression under the severe stress intensity in Cu-exposed roots (figure 5.4+5, table 5.3), but a clear role for antioxidative enzymes and GSH-dependent protection under Cd stress was noticed (table 5.2+3).

In conclusion, for roots, the intensity of Cu stress, combined with its chemical behavior, may stimulate both ROS-mediated and LOX-induced lipid peroxidation. The latter also is important under Cd stress. JA could therefore be included as a component in the signalling pathway (figure 5.6). ROS, produced by NADPH oxidases, are important in intracellular signalling under Cd stress. Moreover, Capone *et al.* (2004) demonstrated an accurate long-distance transmission of ROS from roots to shoots. Therefore H_2O_2 might be important in the intercellular signalling pathway under Cd stress (figure 5.6). Interactions with other plant defence regulators, such as JA, might account for the divergent outcomes of NADPH-dependent ROS signalling (Torres and Dangl 2005).

5.3.2 Oxidative stress as a modulator in Cd and Cu stress in leaves

In leaves, the metal concentrations are much lower (table 5.1). Whereas Cd is partly retained in roots, the retention is nearly complete for Cu in Cu-exposed plants, stressing out the interplay of interorgan signalling molecules in leaf Cu-responses, which was also hypothesized in previous studies (Cuypers *et al.* 2000, Maksymiec 2007). Based on the high *LOX1* gene expression in roots, oxylipins and/or jasmonates could be important molecules in interorgan signalling (figure 5.6) (Kumari *et al.* 2006, Maksymiec 2007). In either metal exposure, the stress intensity in leaves seems mild and hence the signal sensing differs from roots, facing two compartments.

First, whatever the redox relay mechanism, chloroplasts are presumably involved in the perception of a mild stress signal at the very beginning of its action (Kacperska 2004) (figure 5.6). This is supported by our data, since *LOX2* gene expression is profoundly enhanced in leaves after metal exposure as compared to roots in our study (figure 5.3). This enzyme is active in leaves under normal conditions and the enzyme is located in the chloroplasts

(Skorzynska-Polit *et al.* 2006). Changes in the redox state of the chloroplast electron transport chain or of pools of antioxidants (i.e. thioredoxin or glutathione) regulate the expression of several plastome- and nuclear-encoded proteins. Thus, the chloroplast not only provides energy but also may represent a sensor of environmental information, and chloroplast redox signals may help in acclimation of the organism to environmental stresses (Laloi *et al.* 2004, Noctor *et al.* 2007).

Secondly, the involvement of plasma membrane-located NADPH-oxidases under mild stress was confirmed by the gene expression of NADPH oxidases, from which D and F are ABA-dependent; in our experiment they are stimulated under Cd stress (*RBOHF*) and under Cu treatment (*RBOHD*) (figure 5.3). Although the contribution of NADPH oxidases in the ABA-induced stomatal closure is under intense study (Bright *et al.* 2006), it is obvious that more research is needed in order to reveal the precise functions of the different NADPH oxidases and hence be able to attribute a specific role to these enzymes in case of Cd and Cu stress. Interorgan signalling might be important because previous studies have reported an enhancement of ROS, catalyzed by NADPH oxidases, which was influenced by JA (possibly produced in roots) under both stressors (Maksymiec and Krupa 2005, Kumari *et al.* 2006).

Davletova et al. (2005) showed that cytosolic ascorbate peroxidase provides cross-compartment protection to the chloroplast during light stress, and that a *RBOH D* encoded protein might be required for a ROS-dependent signal that maintains high *APX1* levels during light stress. The cytosolic ascorbate peroxidase (APX1) is a central component of the reactive oxygen gene network in *Arabidopsis* (Davletova *et al.* 2005); a key role in the defence mechanism to heavy metal stress and oxidative stress is ascribed to GSH (Ball *et al.* 2004, Semane *et al.* 2007). In leaves of *Arabidopsis*, under both stress conditions, gene expression of *APX1* is induced (figure 5.5). In addition, subcellular localization of the antioxidant components renders the cells a controlled modulation of ROS accumulation spatially and temporally (Mittler *et al.* 2004). As the glutathione redox state (GSSG/GSH) shows an increasing trend under every condition studied, it probably is important in intracellular signal transduction (table 5.2).

5.3.3 Conclusion

In conclusion, the data obtained demonstrate that even under acute environmentally realistic metal concentrations, the signal sensing and hence the cellular response is strongly dependent on the stress intensity and the chemical properties of the metal applied. Clearly ROS-mediated damage as well as signalling is involved under metal stress, but it is becoming increasingly evident that signalling mechanisms in plants often do not operate alone but that extensive cross-talk occurs between signal transduction pathways (Bright *et al.* 2006). Notwithstanding our increasing understanding of signal transduction cascades (Neill *et al.* 2002, Jonak *et al.* 2004, Maksymiec 2007, Yeh *et al.* 2007) (figure 5.6), information on signal transduction pathways after Cd and Cu exposure remains rather fragmentary. Therefore integration of direct metal-induced regulation and ROS-mediated regulation with components of inter- and intracellular signal transduction pathways are important topics for further investigation.

Chapter 6

Oxidative stress related responses after exposure to cadmium and copper in a multipollution context

Karen Smeets, Kelly Opdenakker, Tony Remans, Suzy Van Sanden, Frank Van Belleghem, Brahim Semane, Nele Horemans, Yves Guisez, Jaco Vangronsveld, Ann Cuypers. Oxidative stress related responses at transcriptional and enzymatic level after exposure to Cd or Cu and in a multipollution context. *Submitted*.

Abstract

The physiological effects of Cd and Cu were highlighted in several studies over the last years. At the cellular level, oxidative stress has been reported as a common mechanism in both stress situations. Nevertheless, because of differences in their redox-related properties, the origin of the stress and regulation of these effects can be very different. Our results show a specific Cd-related induction of NADPH oxidases, whereas both metals induce lipid peroxidation via the activation of lipoxygenases. Regarding the antioxidative defence system, metal-specific patterns of SODs (superoxide dismutase) were detected, whereas the gene expression level of the H_2O_2-quenching enzymes was equally induced by both metals.

Because monometallic exposure is very unusual in real world situations, the metal-specific effects were compared with the mechanisms induced when the plants are exposed to both metals simultaneously. A combined exposure to Cd and Cu enhanced some of the induced effects that were induced when only one metal was applied to the medium. Other specific monometallically induced effects, such as a *CSD2* downregulation due to Cd, were also sustained in a multipollution context, irrespective of the other monometallic effect on this. Furthermore, specific multipollution effects were unravelled, as *FSD1* upregulation in the leaves was only significant when both Cu and Cd were applied. Further relationships between these treatments and the common and specific stress induction mechanisms are discussed within this manuscript.

6.1 Introduction

At increased exposure levels, Cd and Cu are known to induce stress effects in all organisms (Lagriffoul *et al.* 1998, Clijsters *et al.* 1999, Fargasova 2001). Whereas Cd is a non-essential trace element and is toxic at even low concentrations, a minimal amount of Cu is needed to survive. Nevertheless, also Cu toxicity remains a growing problem because of the high concentrations locally found in the environment.

At the cellular level, Cd as well as Cu induce oxidative stress (Cuypers *et al.* 2000, Smeets *et al.* 2005, Martins and Mourato 2006). Oxidative stress is a disturbance of the cellular redox status which is often observed in stress situations. ROS (reactive oxygen species) are also produced under normal circumstances and plant cells posses a well equipped antioxidative defence system to maintain the redox equilibrium (Mittler *et al.* 2004, Bhattacharjee 2005, Foyer and Noctor 2005a). In non-stress conditions, ROS and other oxidants are balanced against the antioxidative defence system which is composed of enzymes as well as metabolites (Halliwell 2006). All subcellular compartments contain specific antioxidative enzymes or metabolites which are coordinated and controlled by underlying regulatory mechanisms. Enzymes such as superoxide dismutases (SOD) are able to neutralize superoxide ($O_2^{\circ -}$), and catalases (CAT) and peroxidases (PX) have a role in quenching hydrogen peroxide (H_2O_2).

In stress-conditions, this redox equilibrium is disturbed and organisms react by activating their antioxidative defence system (Mittler 2002). Increases in antioxidative enzyme activities have already been described for Cd and Cu toxicity (Gupta *et al.* 1999, Vitoria *et al.* 2001, Drazkiewicz *et al.* 2003, Drazkiewicz *et al.* 2004, Smeets *et al.* 2005). Cu is a redox-active metal and therefore is able to produce ROS directly via Fenton and Haber-Weiss reactions. Cd, on the other hand, does not have redox-active properties and probably induces oxidative stress via indirect mechanisms such as interaction with the antioxidative defence, disruption of the electron transport and induction of lipid peroxidation. It is already indicated that Cd induces lipoxygenases, which can result in enzymatic lipid peroxidation (Somashekaraiah *et al.* 1992, Skorzynska-Polit and Krupa 2006, Skorzynska-Polit *et al.* 2006, Smeets *et al. in press*).

Another hallmark of Cd-induced oxidative stress is the production of H_2O_2 (as a result of superoxide dismutation or independent of this process) (Cho and Seo 2005, Ortega-Villasante *et al.* 2007). H_2O_2 can modulate the expression of various genes, such as transcription factors, antioxidative genes and other stress-related genes (Kovtun *et al.* 2000, Neill *et al.* 2002). In pea leaves, NADPH oxidase activity was shown to be the main source of H_2O_2 generation (via $O_2^{\circ-}$) after Cd exposure (Romero-Puertas *et al.* 2004, Maksymiec and Krupa 2005, Torres and Dangl 2005). Also inhibition effects on the antioxidative defence system, for example on CuZnSOD, were described under Cd toxicity (Sandalio *et al.* 2001, Drazkiewicz *et al.* 2007).

In summary, oxidative stress presumably plays an important role in the cellular metal toxicity. The aim of this study was to reveal the underlying mechanisms of this oxidative stress induction. Because of the differences in redox-active properties, it is important to gain more information regarding the different mechanisms involved in Cd and Cu toxicity. Furthermore, organisms are never exposed to one metal only and it useful to know which mechanisms are common or rather specific stress effects. Therefore, the effects of Cd and Cu toxicity were analysed in this study and compared with the toxicity mechanisms induced when 3-week-old *Arabidopsis thaliana* plants are exposed to a combination of both metals.

6.2 Results

Three-week-old *Arabidopsis thaliana* seedlings were exposed for 24h to 10 µM $CdSO_4$, 10 µM $CuSO_4$ or to a combination of both metals. No significant changes were observed in the growth parameters of the leaves. In the roots, however, significant growth reductions were observed in the Cu-treated plants as well as in the plants simultaneously exposed to Cd and Cu (results not shown). To confirm the non-lethality of the used Cu concentration, an additional growth experiment was performed. The plants survived longer exposure times (1-2 weeks even to 10 µM $CuSO_4$). Although both root and shoot growth were significantly reduced with a factor 2.3 and 2.9 respectively after 1 week of exposure to 10 µM Cu, neither chlorosis nor necrosis could be detected. The older leaves did show stress effects such as anthocyanin accumulation. After two weeks of exposure, growth was reduced with a factor 6.3 and 8.5 in respectively

roots and leaves. At that moment, all the leaves were intensely anthocyanous-coloured, but necrotic lesions were not observed.

6.2.1 Elemental profile

The root elemental profile of 3-week-old *Arabidopsis thaliana* plants exposed to Cd showed significant increases in Zn and Cd contents (table 6.1). As could be expected, the Cd content also significantly increased in the roots of the plants exposed to both metals, albeit less pronounced then in the roots of the monometallic exposed plants. A similar trend was measured for the root Cu content in the Cu-exposed plants. Decreases in K, Mn and Zn contents were observed in the plants exposed to both metals, whereas in Cu-treated plants only K significantly decreased in the roots (table 6.1). No effects were noticed in the Mg, Ca and Fe content of the metal-exposed plants

***Table 6.1**: Element concentration (mg kg^{-1} DW) after 24h exposure of 3-week-old Arabidopsis thaliana seedlings to 10 µM $CdSO_4$, 10 µM $CuSO_4$ or both during 24h. Values are mean ± SE of 4 independent biological replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

	ROOTS			
Elements	Control	Cd	Cu	Cd+Cu
K	36106±801	34556±669	3207±369***	2844±2089***
Mn	450±29	355±32	344±86	213±42***
Cu	82±47	179±83	5462±197***	5645±214***
Zn	154±16	237±18*	102±6	117±6*
Cd	< 0.8	5899±614***	< 0.8	2143±68***

	LEAVES			
Elements	Control	Cd	Cu	Cd+Cu
Mg	9723±165	8620±200	7979±98	7132±115***
K	26042±617	25521±736	24458±476**	24985±20**
Ca	31774±669	28700±465	24954±422	20588±485***
Mn	217±8	194±9	168±3**	110±4***
Cu	11±3	8±0.4	27±4***	19±2***
Zn	76±9	63±4	52±2	38±3*
Cd	< 0.8	693±42***	< 0.8	13±1***

In the leaves of the same plants, the Cu content significantly increased in plants exposed to 10 µM $CuSO_4$, as did the Cd content in plants treated with 10 µM $CdSO_4$ (table 6.1). In plants simultaneously exposed to both metals, the leaf Cd and Cu contents were also significantly increased, but not as strong as the

monometallic treated plants. In leaves of the Cd-exposed plants, the nutrient acquisition was unaltered. Exposure to 10 µM $CuSO_4$, as well as a combined exposure to 10 µM Cu and Cd resulted in significant decreases of K, Mn and Zn (table 6.1). In addition, plants exposed to Cu and Cd together showed significant decreases of leaf Mg and Ca content

6.2.2 Lipid peroxidation

The level of lipid peroxidation was determined by means of the TBArm content in the roots and leaves of plants exposed to Cd, Cu or both elements combined. Significant increases in TBArm content of the roots and leaves were observed in all treatments as compared with the control plants (figure 5.1). In the roots, a synergistic effect of both metals was detected in the plants exposed to a combination of both metals. In the leaves of these plants, no increase in lipid peroxidation was observed as compared to the leaves of the Cu-treated plants.

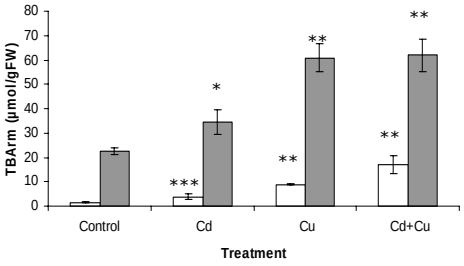


Figure 6.1*: Lipid peroxidation measurement was based on the amount of TBA reactive metabolites (µmol g-1 FW) and was analyzed in roots (white) and leaves (grey) of 3-week-old Arabidopsis seedlings exposed to 10 µM Cd or 10 µM Cu or both Cd+Cu over a 24 h period or grown under control conditions. Values are mean ± S.E. of at least 6 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1).*

6.2.3 Gene expression

Transcript levels of several ROS-producing and antioxidative enzymes were determined in the roots and leaves of metal-exposed plants. In the roots, decreases in transcript levels of ROS-producing and antioxidative enzymes were observed in plants treated with Cu or with the combination of Cu and Cd, except for *LOX1* (lipoxygenase 1) (table 6.2). These transcript levels were significantly increased in all metal-exposed plants. In contrast, when plants were exposed to Cd only, root transcript levels of the ROS producing enzymes *LOX1*, *RBOHC* (respiratory burst oxidase homolog C) and *RBOHD* and *RBOHF* were also significantly upregulated. In addition, gene expression levels of antioxidative enzymes such as *FSD1* (iron superoxide dismutase 1), *CAT1* (catalase 1), *APX1*

(ascorbate peroxidase 1) and *GR1 (*glutathione reductase 1) were significantly increased in the roots of Cd-exposed plants (table 6.2).

In the leaves, increases in transcript abundance of ROS-producing enzymes such as *LOX1*, *LOX2* and *RBOHD* were observed under all exposures (table 6.2). *RBOHC* and *RBOHD* were also significantly increased in the leaves of the plants exposed to Cd or to the combination of both metals. The mRNA level of the antioxidative enzymes *CAT1*, *APX1* and *GR1* also increased in all three exposures. *MSD1* (manganese superoxide dismutase 1) expression was only significantly increased in the leaves of the plants exposed to Cu or to the combination of both metals. Interestingly, metal-specific expression was observed in leaves for *FSD1, CSD1* and *CSD2* (copper zinc superoxide dismutase 1 and 2). Although upregulated in response to Cd+Cu exposure, *FSD1* was downregulated when plants were exposed to Cu only. Superoxide dismutase genes *CSD1* and *CSD2*, were both downregulated in the leaves of the Cd-exposed plants (albeit not significant for *CSD1*), and *CSD1* was upregulated in the leaves of the plants exposed to Cu. When a combination of Cd and Cu was supplied, *CSD2* transcript levels decreased (like in Cd-exposed plants), whereas *CSD1* levels increased (like in Cu-exposed plants).

6.2.4 Enzyme activities

In addition to the gene expression measurements, activities of antioxidative enzymes such as superoxide dismutase (SOD), syringaldazine peroxidase (SPX), guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were measured in the roots and leaves of 3-week-old *Arabidopsis thaliana* plants exposed to 10 µM $CdSO_4$, 10 µM $CuSO_4$ or the combination of 10 µM $CdSO_4$ and 10 µM $CuSO_4$ during 24h (table 6.3).

In the roots, only GPX activity increased significantly ($p<0.1$) in the Cd-exposed plants. Furthermore, a strong decrease of activities was noticed in the Cu-exposed roots for CAT and APX, whereas there were no significant effects on the activities of other measured enzymes. When both metals were supplied to the plants, a significant decrease in GR activity and a significant increase in GPX activity was noticed in the roots.

Table 6.2: *Transcript level of ROS-producing and antioxidative enzymes, expressed relative to the control, in the roots and leaves of 3-week-old A. thaliana seedlings exposed to 10 µM $CdSO_4$, 10 µM $CuSO_4$ or both during 24h. Each point represents the mean of five biological replicates ± SE (significance levels : ■ and ■ : p<0.01; ■ and ■ : p<0.05 for induction and inhibition respectively).*

	ROOTS				LEAVES			
Gene	Control	Cadmium	Copper	Cd+Cu	Control	Cadmium	Copper	Cd+Cu
LOX1	1±0.02	38.98±8.28	11.10±3.07	2.31±0.39	1±0.26	3.66±0.62	2.62±0.41	8.72±2.52
LOX2	1±0.54	2.53±0.29	0.66±0.30	0.33±0.07	1±0.16	4.30±1.33	4.38±1.29	11.1±2.54
RBOHC	1±0.338	4.30±0.21	0.05±0.01	0.02±0.005	1±0.44	16.57±1.10	1.67±1.53	11.84±2.56
RBOHD	1±0.23	5.49±0.21	0.69±0.19	0.51±0.07	1±0.26	2.41±0.51	3.26±0.46	4±0.72
RBOHF	1±0.44	6.91±0.28	0.13±0.009	0.1±0.01	1±0.12	4.81±0.98	1.08±0.19	3.95±0.54
CSD1	1±0.18	1.30±0.31	0.14±0.04	0.08±0.03	1±0.11	0.78±0.26	3.36±0.55	4.42±1.31
CSD2	1±0.12	1.24±0.36	0.05±0.02	0.07±0.005	1±0.18	0.31±0.22	1.30±0.39	0.79±0.15
FSD1	1±0.29	5.88±0.46	0.002±0.001	0.03±0.007	1±0.13	1.13±0.20	0.19±0.04	6.16±5.11
MSD1	1±0.13	2.13±0.07	0.09±0.03	0.10±0.03	1±0.12	1.13±0.23	1.63±0.19	2.08±0.29
CAT1	1±0.13	2.14±0.21	0.92±0.24	0.59±0.08	1±0.26	2.29±0.62	2.04±0.41	2.13±0.21
APX1	1±0.15	1.83±0.07	0.05±0.02	0.12±0.005	1±0.17	2.56±0.57	2.51±0.42	2,73±0,54
GR1	1±0.14	5.90±0.15	0.07±0.07	0.22±0.04	1±0.23	2.56±0.59	1.83±0.39	4.28±0.68

In the leaves, significant changes were observed in the Cd-exposed plants. An increasing pattern in the activities of GPX, CAT, APX and GR was detected as compared to the control plants. The same increasing tendency was noticed in the plants exposed to the combination of Cd for SPX and APX activities. In leaves of Cu-exposed plants, no significant changes of enzyme activities were observed.

Table 6.3*: Enzyme activities (mU g^{-1} FW) in the leaves of 3-week-old A. thaliana seedlings exposed to 10 µM $CdSO_4$, 10 µM $CuSO_4$ or both during 24h. Values are mean ± SE of 4 independent biological replicates (significance levels : * : p-value<0.1; ** : p-value<0.05; *** : p-value<0.01).*

	ROOTS			
Enzymes	Control	Cd	Cu	Cd+Cu
SOD	130862±105745	126781±10230	116382±9341	124466±8916
SPX	6199±1016	6613±506	7188±778	5927±409
GPX	4178±1207	7648±1674*	5022±4046	7043±510*
CAT	123±26	132±25	40±33***	71±17
APX	6107±1016	7867±812	1602±605***	3281±450
GR	19±3	25±1	17±0.7	12±2**

	LEAVES			
Enzymes	Control	Cd	Cu	Cd+Cu
SOD	121081±17054	124723±6928	139016±7108	148628±13229
SPX	3241±363	7796±1276	4243±666	7737±1521*
GPX	1032±82	2532±664*	1283±215	1285±217
CAT	381±63	443±72*	314±84	720±81
APX	17510±3021	23357±7959*	14098±398	29232±5396*
GR	79±7	100±5*	85±6	100±7

6.2.5 Ascorbate and glutathione content

The antioxidative metabolite content was determined in the roots and leaves of plants exposed to Cd, Cu or the combination of both elements (figure 6.2).

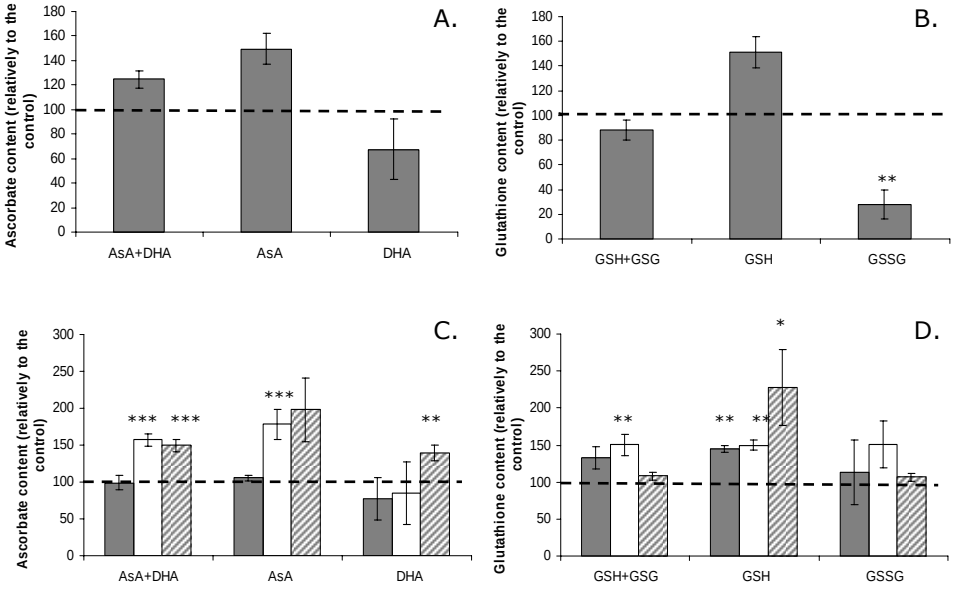


Figure 6.2: *Metabolite concentrations (relative to the control plants = 100%) in roots (A+B) and leaves (C+D) of 3-week-old Arabidopsis seedlings exposed to 10 µM Cd (grey) or Cu (white) or both Cd+Cu (grey+white) over a 24h period or grown under control conditions. In the roots, the metabolite levels of the plants treated with Cu and Cd+Cu were below detection limit. (AsA: reduced ascorbate, DHA: dehydroascorbate; GSH: reduced glutathione, GSSG: glutathione disulfide). Values are mean ± S.E. of at least 3 biological independent replicates (significance levels: ***: p<0.01; **: p<0.05; *: p<0.1.*

In the roots of these metal-exposed plants, slight increases in total ascorbate and AsA content were noticed when exposed to Cd (figure 6.2A), whereas in the Cu and Cd+Cu-exposed plants strong decreases were observed in total ascorbate, AsA and DHA contents (results not shown). In the plants exposed to

Cd, a strong enhancement in GSH content was observed, whereas the GSSG content was significantly reduced (figure 6.2B). The other exposures caused significant decreases of glutathione contents in the roots (results not shown).
In the leaves, significant increases were noticed in the total ascorbate and in the total glutathione content of the Cu and Cd+Cu exposed plants. In the plants exposed to 10 µM $CuSO_4$, the AsA content was significantly increased whereas in the Cd+Cu-exposed plants an increase in DHA was observed (figure 6.2C). Furthermore, the GSH level was significantly increased in all treatments, whereas no significant effects were noticed in the GSSG content (figure 6.2D).

6.3 Discussion

Both Cd and Cu are known to induce oxidative stress in organisms exposed to either metal (Cuypers *et al.* 2002, Ortega-Villasante *et al.* 2005, Rodriguez-Serrano *et al.* 2006). Whereas Cu is a redox-active metal and by consequence is able to cause ROS production directly, Cd induces oxidative stress via indirect mechanisms such as an inhibition of antioxidative defence systems, or via the activation of ROS-producing enzymes like NADPH oxidases (Romero-Puertas *et al.* 2004, Chaoui and El Ferjani 2005). Because organisms are almost always exposed in a multipollution context, oxidative stress related responses for single element exposures were compared with these occurring after exposure to the combination of both metals. In this way a better insight in common but also metal-specific induced oxidative stress responses was aimed.
A relative reduction in root biomass as well as decreased concentrations of elements such as K (and to a lesser extent Mn and Zn) were detected in roots of Cu-exposed plants. The K content decreased significantly with a factor 10, indicating membrane leakage. Peroxidative degradation of membrane lipids (figure 6.1) might be involved in the membrane permeability change after exposure either to Cu alone or in combination with Cd. This lipid peroxidation probably is mediated by ROS that are produced as a direct consequence of the redox-active properties of Cu. In the roots of Cd-exposed plants, a significant upregulation of *LOX1* was observed (table 6.2), which might be important in Cd-induced lipid peroxidation. Besides causing lipid peroxidation, lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids producing hydroperoxy fatty acids which can be subsequently metabolized via several secondary

pathways to produce bioactive compounds such as jasmonate and oxylipins (Porta and Rocha-Sosa 2002). Because *LOX1* gene expression was significantly increased in all metal treatments investigated, it might have a role in a root to shoot signalling via the production of oxylipines and jasmonates under metal stress.

The metal-induced oxidative stress in the roots differed as a direct consequence of the chemical behaviour of Cu and Cd, but also changes in ROS producing and scavenging enzymes were noticed (table 6.2+3). The genes for NADPH oxidases, enzymes that are a potential source for ROS, were upregulated in the roots of the Cd-exposed plants but they were transcriptionally downregulated in the roots of Cu-exposed plants in a single or multipollution context. The antioxidative defence was downregulated in Cu-exposed plants, whereas it was significantly upregulated under Cd-treatment. When studying the root responses in the combination of metals in comparison with the monometallic exposed plants, they were very similar to the responses in Cu-exposed roots.

The leaf parameters showed even more interesting markers for each specific metal treatment. Significant increases in Cd and Cu content were detected in the leaves of the plants exposed to respectively Cd and Cu. In the leaves of the plants exposed to both metals, however, a relative reduction (as compared to monometallic exposed plants) in Cd and Cu contents was observed. As this reduction was more pronounced for Cd, root-to-shoot transport might be disturbed due to the strong phytotoxic effect of Cu in the roots.

In the leaves, NADPH oxidases obviously play a highly important role in the Cd-induced oxidative stress (table 6.2), and H_2O_2 might be important during Cd stress as increases in gene expression of H_2O_2-quenching enzymes such as CAT1, APX1 and GR1 were observed (table 6.2). In the leaves of Cu-exposed plants, transcriptional upregulations of H_2O_2-quenching enzymes were also detected, but no differences were noticed in the transcript abundance of the ROS producing *RBOHC* and *RBOHF* (table 6.2). This again suggests that also in the leaves, the production of ROS caused by excess Cu was a direct consequence of its redox-active properties. Interestingly, in the leaves of plants exposed to a combination of both metals, the gene expression level of NADPH oxidases was still highly upregulated, like it was for the Cd-exposed plants. This may indicate that at similar concentrations of Cd and Cu, the dominant effect on NADPH

oxidase gene expression in the leaves is exerted by Cd, whereas in the roots it is exerted by Cu.

Lipoxygenases are involved in Cd as well as in Cu stress. Both *LOX1* and *LOX2* were significantly increased in the leaves of metal-exposed plants, but a more pronounced upregulation was detected in the leaves of plants exposed to a combination of both metals. In these plants lower Cd and Cu leaf concentrations were observed (as compared to the single metal-exposed plants). Therefore a possible role for oxylipins/jasmonates in inter- and intracellular signalling can be hypothesized.

Interesting treatment-specific patterns were detected in the gene expression profile of superoxide quenching enzymes. Whereas CuZnSODs are important in the antioxidative defence during Cu-toxicity (table 6.2) (Sunkar *et al.* 2006), an opposite effect on both *CSD1* and *CSD2* expression was detected under Cd-stress (table 6.2). Moreover, a specific *SOD* expression pattern was observed in the multipollution context, with *CSD1*and *MSD1* regulated as in the Cu treatment, *CSD2* regulated as in the Cd treatment (*CSD2*), and *FSD1* showing an increased expression specific to the combined treatment. At the activity level, no significant changes in total SOD activity were found (table 6.3), but other studies reported isoform-specific activity changes in accordance with our gene expression results (Drazkiewicz *et al.* 2007). Diverse regulation pathways and/or changes in element contents such as Cu and Zn are possible underlying mechanisms behind these discrepancies (Weber *et al.* 2006, Cohu and Pilon 2007).

It can be concluded that lipoxygenases and the generation of H_2O_2 probably play an important role in both Cu and Cd toxicity, but different mechanisms are responsible for the H_2O_2 production. Whereas NADPH oxidases are induced as key components in the Cd-dependent H_2O_2 production, Cu because of its redox-active properties is able to produce ROS directly. At the antioxidative side, significant increases in gene expression of some antioxidative genes were observed for all exposures, and increased activities of several H_2O_2-quenching enzymes was noticed in the leaves of the plants exposed to Cd, or to both Cd+Cu. Treatment-specific effects were observed in the gene expression of superoxide-quenching enzymes. Some of these showed a metal-specific effect that was consistent even in a multipollution context, whereas *FSD1* showed a

multipollution specific induction. To explain these results, the specificity of the underlying stress mechanisms need to be studied, which are influenced by metal concentration, metal speciation and time of exposure.

Chapter 7

Reactive oxygen species contribute to the induction of signal transduction pathways in the roots of *Arabidopsis thaliana* during cadmium exposure

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Abstract

During the last years, oxidative stress has been implicated as an important factor in Cd toxicity. In this study, hydrogen peroxide as well as superoxide were shown to be significantly increased in roots of 3-week-old *Arabidopsis thaliana* seedlings exposed for 24h to 5 or 10 µM Cd. A microarray analysis was performed to reveal the most important Cd-responsive genes after induction of specific reactive oxygen species (ROS) during Cd stress. Among the 26000 genes analyzed, 1639 transcripts significantly differed in the Cd-treated plants. A subset of 145 genes was related to the redox status of the cell, whereas almost 20% was related to signal transduction mechanisms, which included various components of the MAPK-cascades and Ca^{2+}-dependent kinases. Clustering analysis revealed a coordinated regulation response of these signalization cascades at the transcriptional level during Cd stress. In addition, our results suggest a causal role for ROS as a common regulation mechanism and as a missing link between the ROS-related enzymes and the signalling cascades that are triggered during exposure to Cd.

7.1 Introduction

Cd is a non-redox-active metal, capable of provoking oxidative stress, that can lead to physiological damage such as disruption of transport, photosynthesis and transpiration (Hsu and Kao 2005, Maksymiec *et al.* 2007). As described previously, organisms try to counteract this induced oxidative stress by activating their antioxidative defence system at the transcriptional and/or enzymatic level (Chaoui and El Ferjani 2005, Smeets *et al.* 2005, Smeets *et al. in press*). A fundamental role for hydrogen peroxide (H_2O_2) was putted forward, mentioned in different studies concerning Cd toxicity. Increases in H_2O_2 contents were observed after exposure to low and high Cd concentrations and similar stress effects were described when plants were treated with only H_2O_2 (Schutzendubel *et al.* 2002, Romero-Puertas *et al.* 2004, Yang *et al.* 2007). Experiments with inhibitors suggested that NADPH oxidases could be a major source of this H_2O_2 during Cd toxicity (Romero-Puertas *et al.* 2004). Other oxidative stress related effects were also observed under Cd stress. The levels of lipid peroxidation, for example, were several times higher in both roots and leaves of plants treated with Cd (Singh *et al.* 2006, Skorzynska-Polit and Krupa 2006).

Besides their toxic features, ROS (reactive oxygen species) are also capable of modulating signalling networks that control physiological processes and stress responses (Mittler *et al.* 2004, Foyer and Noctor 2005a). In fact, ROS, such as H_2O_2, are ideal signalling molecules as they are small and able to diffuse over short distances. They can influence the expression of a number of genes involved in signal transduction, metabolism, cellular organization, cell rescue, … (Desikan *et al.* 2001, Gadjev *et al.* 2006). Nonetheless, the identity and modes of action of the transmitting signals during Cd stress remain unknown.

ROS can be detected by several cellular components such as ROS receptors and redox-sensitive transcription factors (Mittler *et al.* 2004). Detection of ROS by receptors can result in the release of Ca^{2+} from intracellular stores or in the activation of phospholipases (Bhattacharjee 2005, Wang *et al.* 2006). Former studies already demonstrated a coordinated link between Cd exposure and Ca^{2+} signalling, whether or not via H_2O_2 as an intermediate signalling molecule or as a second messenger produced via a Ca^{2+}-induced oxidative burst (Garnier *et al.*

2006). The generation of ROS, Ca^{2+} signals and the activation of specific phospholipases are thought to activate Ca^{2+}-dependent kinases as well as other signal transduction cascades including the MAPK (mitogen-activated protein kinase) - pathway (Reddy 2001, Mittler *et al.* 2004). MAPK are one of the largest family of serine-threonine kinases in higher plants that transduce extracellular signals to regulate cellular processes such as cell division, hormone production and defence mechanisms (Mishra *et al.* 2006). MAPK can be activated in a matter of minutes, and *de novo* translation is not required.

In summary, the ROS network appears to play a key role in the activation and regulation of physiological processes and defence mechanisms. Because of their toxic properties, ROS concentrations need to be tightly regulated and controlled by production and scavenging. Figure 7.1 summarizes known relations of ROS-producing, -scavenging and regulatory pathways. In this study, we tried to unravel which specific ROS, ROS-producing components, signalling cascades and antioxidative proteins were induced in the roots of *Arabidopsis thaliana* due to Cd exposure. To gain better insight in the molecular mechanisms behind Cd stress, a total screening at the transcriptional level was performed.

7.2 Results

7.2.1 Morphological effects of Cd in the roots

In the current study, neither growth reduction nor severe morphological effects at the macroscopic level were observed in the roots of *Arabidopsis thaliana* plants after 24h of exposure to any of the Cd concentrations. A significant reduction of the cell wall thickness of xylem vessels was found after exposure to 10 µM $CdSO_4$ (figure 7.2). Based on electron microscopic measurements of 5 different roots/treatment, a significant decrease from 739±19 nm to 645±20 nm was observed ($p<0.05$). Interestingly, expression levels of two phenylalanine ammonia-lyase (*PAL1* and *PAL2)* genes were significantly decreased (table 7.2). PAL catalyzes the first step in phenylpropanoid synthesis, which is an intermediate in the construction of xylem vessels.

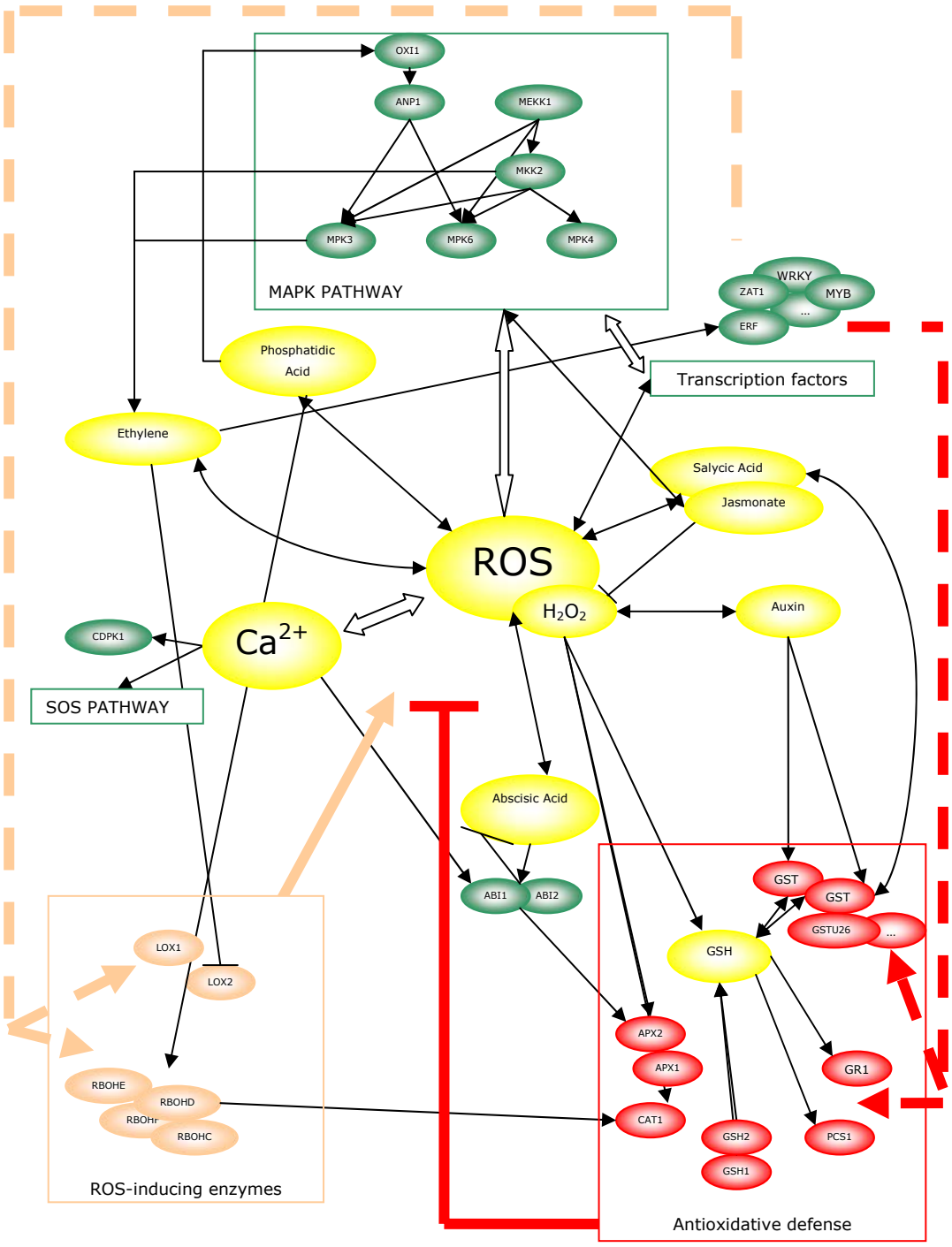

OXI1
ANP1
MEKK1
MKK2
MPK3
MPK6
MPK4
MAPK PATHWAY
WRKY
ZAT1
MYB
...
ERF
Transcription factors
Phosphatidic Acid
Ethylene
Salycic Acid
Jasmonate
ROS
H_2O_2
Auxin
Ca^{2+}
CDPK1
SOS PATHWAY
Abscisic Acid
ABI1
ABI2
GST
GST
GSTU26
...
GSH
LOX1
LOX2
APX2
APX1
GR1
CAT1
GSH2
GSH1
PCS1
RBOHE
RBOHD
RBOHF
RBOHC
ROS-inducing enzymes
Antioxidative defense

***Figure 7.1** (p88): Generalized model of existing pathways between ROS, oxidative stress related mechanisms and signal transduction cascades, based on the literature. In a stress situation, ROS, such as H_2O_2, can activate signal transduction pathways such as the MAPK cascade and Ca^{2+} related cascades that, for their part, are able to activate different transcription factors. ROS can also directly activate these transcription factors that regulate ROS-scavenging (red arrow) and ROS-producing (pink arrow) pathways. In addition, ROS-scavenging and ROS-producing enzymes too can be directly regulated by ROS, on both (post)transcriptional and (post)translational level. (ABI= ABA insensitive protein phoasphatase 2C), ACS= 1-aminocyclopropane-1-carboxylate (ACC) synthase, ANP= NPK-related protein kinase, APX= ascorbate peroxidase, CAT= catalase, CDPK= Ca^{2+}-dependent kinase, CSD= copper zinc superoxide dismutase, GR= glutathione reductase, GSH= glutathione/ glutathione synthetase, GST= glutathione-S-transferase, FSD= iron superoxide dismutase, LOX= lipoxygenase, MAPK= MAPkinase, MKK= MAPK kinase, MEKK1= MAPK kinase kinase, OXI1= oxidative signal inducible kinase 1, RBOH= respiratory burst oxidase homolog, SOS= salt overly sensitive protein). (ROS-producing components = pink/ antioxidative components = red/ signalling components = green)*

7.2.2 ROS and regulation mechanisms during Cd stress

A transcriptome analysis was performed to investigate the cellular response in *Arabidopsis thaliana* roots during Cd toxicity. Among the 26.000 genes analyzed, 1639 transcripts significantly differed in the Cd-exposed plants. A subset of 145 genes was related to the redox status of the cell, whereas almost 20% was related to signal transduction mechanisms (tables 7.1 and 2).

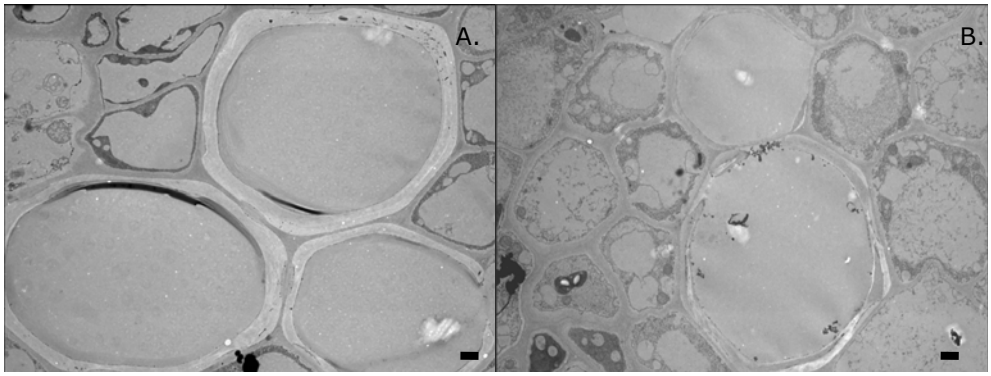


***Figure 7.2:** Electron micrographs demonstrating the effect on the ultrastructure of the xylem vessels in the roots of 3-week-old Arabidopsis seedlings exposed to 0 µM $CdSO_4$ (A) and 10 µM $CdSO_4$ (B) for 24h (bars: 700 nm).*

7.2.2.1 The cellular redox status in Arabidopsis roots is affected after application of Cd

7.2.2.1.1 ROS production

ROS in roots were studied using crude extracts prepared in the presence of a spin trap. The absence of any detectable DEPMPO-adducts in extracts of untreated roots shows that free radicals were not induced by the extraction

procedure itself (figure 7.3). In extracts made from Cd-exposed roots, superoxide radicals were observed in response to both 5 and 10 µM $CdSO_4$, albeit to a smaller extent in the roots of the plants exposed to the lower concentration. Apart from this free radical, levels of a non-radical ROS, H_2O_2, were also significantly increased after exposure to 5 and 10 µM $CdSO_4$ (figure 7.4). As compared to the control plants, H_2O_2 concentrations were a factor 2-2.5 higher in the roots of the Cd-exposed plants. Additional indication for a disturbed redox status came from the analysis of lipid peroxidation using a thiobarbituric acid (TBA) determination (chapter 5: figure 5.1). Only an exposure of 10 µM $CdSO_4$ resulted in a significant increase in lipid peroxidation products in the roots of *Arabidopsis thaliana*.

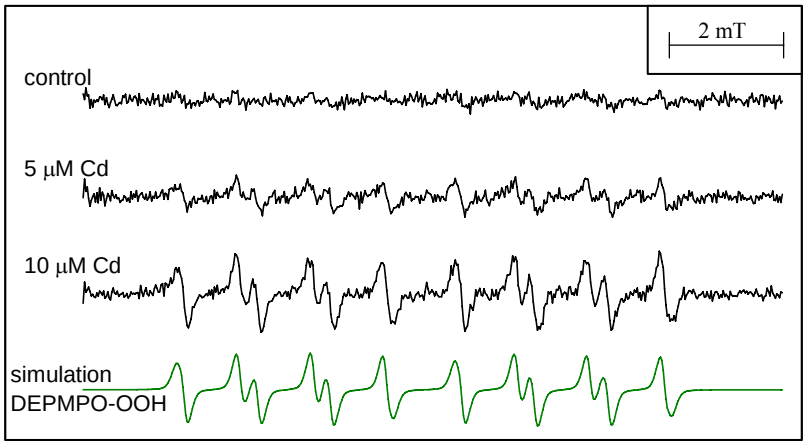


***Figure 7.3**: EPR-spectra of DEPMPO-adducts produced in extracts of Arabidopsis thaliana roots exposed to 0 µM $CdSO_4$, 5 µM $CdSO_4$ and 10 µM $CdSO_4$.*

In order to determine possible sources of this ROS production, gene expression was investigated using Real Time PCR (table 7.2). All NADPH oxidases were transcriptionally induced in the roots of the Cd-exposed plants (table 7.2). The induction of *RBOHD* was significant at an exposure concentration of 10 µM, whereas *RBOHE* was significantly upregulated at all exposure concentrations. *RBOHD*, *RBOHE* and *RBOHF* were also significantly upregulated in the microarray screening (table 7.1). In addition, expression of the cytoplasmic lipoxygenase gene (*LOX1*) was increased with a factor 2 in roots of plants exposed to 2 µM $CdSO_4$, but was tremendously enhanced after exposure to 5 and 10 µM $CdSO_4$. No significant changes were noticed in the transcript level of the plastidic lipoxygenase, LOX2.

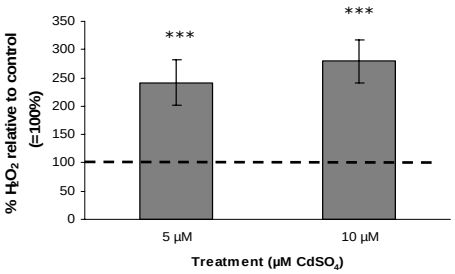


***Figure 7.4**: The H_2O_2 content measured in the roots of 3-week-old Arabidopsis thaliana seedlings exposed to 5 or 10 µM $CdSO_4$ over a 24h period or grown under control conditions (=100%). Values are mean ±S.E. of 3 biological independent replicates (significance levels: ***: p<0.01).*

7.2.2.1.2 ROS scavenging

Several components of the antioxidative defence system were measured at the transcriptional level. The *FSD1* (iron superoxide dimutase 1) gene was transcriptionally upregulated (with a factor 7-9) in the roots of the plants exposed to 5 and 10 µM $CdSO_4$. *CSD1* (copper zinc superoxide dismutase 1) and *CSD2* (copper zinc superoxide dismutase 2) transcription, on the other hand, was diminished under 2 and 5 µM $CdSO_4$ exposure, but was again comparable to the control level at 10 µM. Concerning the H_2O_2-quenching enzymes, a limited downregulation in the expression of the peroxisomal catalase gene (*CAT1*) was noticeable at an exposure to 2 µM $CdSO_4$, whereas a transcriptional induction was observed at higher exposure concentrations. The expression level of *APX1* (ascorbate peroxidase 1) gene was slightly downregulated at all exposure concentrations, whereas the *APX2* (ascorbate peroxidase 2) gene was strongly induced in the roots of Cd-exposed plants (up to 60 fold at higher exposure concentrations).

Furthermore, a central role for glutathione (GSH) during Cd toxicity is expected from the microarray results. Various glutathione-related genes, such as glutathione-S-transferases, glutathione transporters and glutathione-related antioxidative enzymes, differed significantly from those in control plants (table 7.1). *GSH2* (glutathione synthetase 2), *GST6* (glutathione-S-transferase 6) and *MRP4* (multidrug resistance-associated protein 4) were selected for supplementary information related to the role of GSH during Cd stress, as they are important in other stress situations (table 7.2). The patterns of *GSH1* (gamma-gluatmylcysteine synthetase), *GST2* (glutathione-S-transferase 2) and *GSTU26* (glutathione-S-transferase (class tau) 26) agreed very well with the microarray data. *GSH1* was strongly induced at 10 µM $CdSO_4$ (with a factor 6).

Table 7.1 *(next pages): The most important genes relative to the redox balance of the cell, or to signal transduction mechanisms, that showed an upregulation in response to 5 and/or 10 µM $CdSO_4$ (=: stabe, += significant upregulation, ++: significant upregulation relative to 5 µM $CdSO_4$).*

Genes related to the redox status	Atg number	5 µM	10 µM
superoxide dismutase [Fe]	At4g25100	=	+
catalase 1	At1g20630	=	++
catalase 3 (SEN2) almost identical to catalase 3	At1g20620	+	+
glutamate-cysteine ligase / gamma-glutamylcysteine synthetase (GSH1)	At4g23100	=	+
glutathione reductase	At3g24170	=	+
glutathione S-transferase	At1g02920	+	++
glutathione S-transferase	At1g02930	+	++
glutathione S-transferase	At1g17170	+	++
glutathione S-transferase	At1g17180	+	++
microsomal glutathione s-transferase	At1g65820	+	+
glutathione S-transferase	At1g69920	+	++
glutathione S-transferase	At1g74590	+	++
glutathione S-transferase	At2g02930	+	++
glutathione S-transferase	At2g29420	+	+
glutathione S-transferase	At2g29460	+	++
glutathione S-transferase	At3g09270	+	+
glutathione S-transferase	At4g02520	+	+
glutathione S-conjugate ABC transporter (MRP2)	At2g34660	=	+
ABC transporter family protein (MRP3)	At3g13080	+	+
putative similar to glutathione-conjugate transporter AtMRP4	At3g62700	+	+
ABC transporter family protein (MRP8)	At3g13090		
ABC transporter family protein similar to ATP-binding cassette transporter MRP8	At3g13100	+	++
phytochelatin synthase 1 (PCS1)	At5g44070	+	+
phytochelatin synthetase-related	At3g16860	+	++
L-ascorbate oxidase	At4g39830	+	+
monodehydroascorbate reductase	At3g52880	=	+
anionic peroxidase	At1g14540	+	++

peroxidase, putative identical to peroxidase ATP5a	At1g49570	+	+
peroxidase 12 (PER12) (P12) (PRXR6)	At1g71695	+	+
Peroxidase	At2g18150	+	++
Peroxidase	At3g03670	+	++
peroxidase 33 (PER33) (P33) (PRXCA)	At3g49110	=	+
Peroxidase	At3g49120	+	+
peroxidase, putative identical to class III peroxidase ATP38	At4g08770	+	+
peroxidase, putative similar to peroxidase isozyme	At4g08780	+	++
peroxidase, putative identical to peroxidase ATP19a	At4g11290	+	+
Peroxidase	At4g36430	+	++
peroxidase 50 (PER50) (P50) (PRXR2)	At4g37520	+	++
Peroxidase	At5g05340	=	++
Peroxidase	At5g06720	=	+
Peroxidase	At5g06730	+	++
Peroxidase	At5g06730	+	++
peroxidase, putative identical to peroxidase ATP24a	At5g39580	+	+
peroxidase 72 (PER72) (P72) (PRXR8)	At5g66390	+	+
respiratory burst oxidase protein D (RbohD)	At5g47910	+	++
respiratory burst oxidase protein E (RbohE)	At1g19230	+	++
respiratory burst oxidase protein F (RbohF)	At1g64060	=	+
monooxygenase, putative (MO1)	At4g15760	+	++
monooxygenase, putative (MO2)	At4g38540	+	++
glutaredoxin family protein	At1g03850	+	++
glutaredoxin family protein	At5g01420	=	+
thioredoxin H-type 2 (TRX-H-2)	At5g39950	=	+
thioredoxin H-type 5 (TRX-H-5)	At1g45145	+	++
pathogen-responsive alpha-dioxygenase	At3g01420	+	++
electron transfer flavoprotein-ubiquinone oxidoreductase family protein	At2g43400	+	+
alternative oxidase 1a, mitochondrial (AOX1A)	At3g22370	+	++
NADH dehydrogenase-related similar to alternative NADH-dehydrogenase	At4g05020	=	+

Genes related to MAPK signalling	**Atg number**	**5 µM**	**10 µM**
mitogen-activated protein kinase, putative / MAPK, putative (MPK11)	At1g01560	=	+
NPK1-related protein kinase, putative (ANP2) similar to protein kinase	At1g54960	=	+
OXI1, oxidative signal inducible1	At3g25250	+	+
mitogen-activated protein kinase, putative / MAPK, putative (MPK3)	At3g45640	+	+
mitogen-activated protein kinase, putative / MAPK, putative (MPK4)	At4g01370	=	+
mitogen-activated protein kinase, putative similar to MAPKKK10	At4g08470	+	+
mitogen-activated protein kinase, putative / MAPK, putative (MPK5)	At4g11330	+	+
mitogen-activated protein kinase kinase (MAPKK) (MKK2)	At4g29810	+	+
protein kinase family protein, mapkkk19	At5g67080	+	+
MAP kinase phosphatase (MKP1) identical to MAP kinase phosphatase (MKP1)	At3g55270	+	+
Genes related to Ca signalling	**Atg number**	**5 µM**	**10 µM**
calcineurin B-like protein 1 (CBL1)	At4g17615	+	+
calcium-binding EF hand family protein similar to calmodulin-like MSS3	At3g29000	+	+
calcium-binding EF hand family protein	At3g47480	+	++
calcium-binding protein	At4g20780	=	+
calcium-binding EF hand family protein	At5g39670	+	+
calcium-binding protein	At5g44460	+	+
calcium-binding EF-hand protein, putative similar to EF-hand Ca2+-binding protein CCD1	At5g54490	+	+
CBL-interacting protein kinase 11 (CIPK11)	At2g30360	+	+
CBL-interacting protein kinase 1 (CIPK1)	At3g17510	=	+
protein kinase family protein contains protein kinase domain	At4g00330	+	+
calcium-dependent protein kinase, putative / CDPK, putative similar to calcium-dependent protein kinase	At4g09570	+	++
CBL-interacting protein kinase 6 (CIPK6) identical to CBL-interacting protein kinase 6	At4g30960	+	+
calcium-dependent protein kinase family protein / CDPK family protein	At5g66210	+	+
calcium-transporting ATPase 1, plasma membrane-type / Ca(2+)-ATPase isoform 1 (ACA1)	At1g27770	+	++
calcium-transporting ATPase, plasma membrane-type, putative / Ca(2+)-ATPase, putative (ACA12)	At3g63380	+	+
calcium-transporting ATPase, plasma membrane-type, putative / Ca2+-ATPase, putative (ACA10)	At4g29900	=	+
cyclic nucleotide-regulated ion channel (CNGC10) (ACBK1)	At1g01340	=	+
calmodulin-binding protein similar to calmodulin-binding protein TCB60	At1g73800	+	+

ethylene-responsive calmodulin-binding protein	At2g22300	+	++
calmodulin-binding family protein	At2g26190	+	+
touch-responsive protein / calmodulin-related protein 3, touch-induced (TCH3)	At2g41100	+	++
calmodulin-binding family protein	At3g13600	=	+
calmodulin-9 (CAM9) identical to calmodulin 9	At3g51920	+	+
calmodulin-binding family protein	At3g52870	+	++
calmodulin-binding family protein	At4g33050	+	++
calmodulin-binding family protein	At4g33050	+	+
calmodulin-binding protein similar to anther ethylene-upregulated calmodulin-binding protein ER1	At5g09410	=	++
calmodulin-binding protein	At5g26920	+	++
protein phosphatase 2C ABI1 / PP2C ABI1 / abscisic acid-insensitive 1 (ABI1)	At4g26080	=	+
Genes related to other signal transduction pathways	**Atg number**	**5 µM**	**10 µM**
phospholipase D gamma 3 / PLD gamma 3 (PLDGAMMA3) identical to phospholipase D gamma 3	At4g11840	+	+
1-aminocyclopropane-1-carboxylate oxidase / ACC oxidase / ethylene-forming enzyme (ACO) (EAT1)	At1g05010	+	++
1-aminocyclopropane-1-carboxylate oxidase, putative / ACC oxidase, putative nearly identical to ACC oxidase (ACC ox1)	At1g62380	+	+
1-aminocyclopropane-1-carboxylate synthase 6 / ACC synthase 6 (ACS6)	At4g11280	+	++
1-aminocyclopropane-1-carboxylate synthase 2 / ACC synthase 2 (ACS2)	At1g01480	=	+
ethylene-responsive factor, putative Similar to Nicotiana EREBP-3	At1g04370	+	++
ERF domain protein 11 (ERF11)	At1g28370	+	+
ethylene-responsive factor, putative similar to ethylene response factor 1	At2g31230	+	++
ethylene-responsive element-binding protein, putative	At2g44840	+	+
ethylene response factor 1 (ERF1)	At3g23240	+	+
ethylene-responsive element-binding protein	At4g17490	=	+
ethylene-responsive element-binding protein 1 (ERF1)	At4g17500	+	++
ethylene-responsive element-binding factor 2 (ERF2)	At5g47220	+	+

GST2 expression was significantly elevated under all exposure concentrations, and 25-fold increase was observed at the highest exposure concentrations. The expression level of the *GSTU26* gene was significantly downregulated whereas no significant changes were observed in the transcriptional level of *GST6*. In summary, 17 GST genes were differentially expressed, but total GST activity did not show significant changes (results not shown). In addition, several glutathione conjugate transporters were significantly upregulated during Cd-stress (tables 7.1 and 2).

7.2.2.2 Signal transduction pathways upregulated during Cd stress

After the microarray screening, 302 genes were classified as important in regulation and/or signal transduction cascades during Cd stress. Gene expression of 105 transcription factors was significantly induced, among which transcription factors of the WRKY and MYB families. Several transcription factors and hormone responsive proteins related to auxin and ethylene signalling also differed significantly after Cd exposure. Especially the ethylene pathway seemed of great importance, because of an additional upregulation of *ACS2* (1-aminocyclopropane-1-carboxylate synthase 2), *ACS6* and *ACC oxidase*, key enzymes in the ethylene biosynthesis. Table 7.1 shows the most important significant increases perceived from this microarray screening.

7.2.2.2.1 Transcript profiles of MAPK-related genes

Ten genes related to the MAPK-pathway were transcriptionally increased in the microarray screening (table 7.1). The gene expression patterns of *OXI1* (oxidative signal inducible 1), *ANP2* (NPK1-related protein kinase), *MKK2* (MAPkinase kinase 2), *MPK3* (MAPkinase 3) and *MPK4* (MAPkinase 4) were confirmed by means of Real Time PCR (table 7.2). Significant differences were mainly found at the highest exposure concentration (10 µM $CdSO_4$). The expression level of *OXI1* and *MKK2* genes was lowered at an exposure of 2 µM $CdSO_4$, but these differences were not significant. Additionally, the transcript levels of *ANP1, MEKK1* and *MPK6* were measured. No significant differences were found in *MPK6* expression. *ANP1* and *MEKK1*, on the other hand, were transcriptionally increased at the higher exposure concentrations, albeit only significant at the highest Cd concentration.

Gene	Control	2 µM Cd	5 µM Cd	10 µM Cd
Gene expression profile of ROS PRODUCING ENZYMES				
LOX1	1.00±0.25	2.33±0.25**	14.97±3.76***	51.78±11.74***
LOX2	1.00±0.24	1.43±0.88	0.27±0.10	1.28±0.40
RBOHC	1.00±0.25	1.05±0.43	1.22±0.47	1.18±0.29
RBOHD	100±0.23	1.28±0.67	2.31±0.67	5.08±0.93***
RBOHE	1.00±0.40	3.08±1.12*	2.76±1.00*	5.20±2.15**
RBOHF	1.00±0.45	1.25±0.23	1.69±0.51	1.12±0.24
Gene expression profile of ANTIOXIDATIVE ENZYMES				
CSD1	1.00±0.23	0.52±0.12*	0.46±0.12**	0.96±0.15
CSD2	1.00±0.21	0.56±0.13	0.48±0.11	0.83±0.21
FSD1	1.00±0.22	1.49±0.47	7.87±2.56**	9.33±1.21***
CAT1	1.00±0.31	0.61±0.15	1.56±0.35	5.34±1.06**
APX1	1.00±0.31	0.65±0.20	0.63±0.19	0.91±0.18
APX2	1.00±0.28	1.56±0.37	5.87±1.31***	63.72±16.69***
Gene expression profile of GLUTATHION RELATED PROTEINS				
GSH1	1.00±0.72	0.40±1.78	0.79±0.20	6.02±2.13***
GSH2	1.00±0.42	1.11±0.52	0.23±0.09**	0.34±0.11**
GST2	1.00±0.30	2.79±0.70*	24.61±13.36***	25.41±10.35***
GST6	1.00±0.29	0.92±0.38	0.77±0.38	1.23±0.42
GSTU26	1.00±0.16	0.75±0.24	0.44±0.17**	0.52±0.33
MRP4	1.00±0.21	1.15±0.27	2.64±0.92**	2.65±0.61***
Gene expression profile of ENZYMES involved in SIGNAL TRANSDUCTION				
OXI1	1.00±0.22	0.59±0.28	1.38±0.34	1.84±0.56
ANP1	1.00±0.28	1.24±0.29	2.04±0.85	2.40±0.45**
ANP2	1.00±0.13	1.26±0.38	2.18±0.71	5.26±1.41**
MEKK1	1.00±0.22	1.02±0.29	1.07±0.37	2.16±0.35
MKK2	1.00±0.13	0.67±0.26	0.90±0.15	2.46±1.23
MPK3	1.00±0.22	1.03±0.34	1.16±0.32	2.71±0.46***
MPK4	1.00±0.19	1.33±0.33	2.81±1.63	3.78±1.98
MPK6	1.00±0.33	0.88±0.25	1.11±0.51	1.10±0.24
CDPK1	1.00±0.24	0.98±0.22	1.57±0.53	3.06±0.57***
SOS3	1.00±0.33	1.20±0.29	2.28±0.63**	5.55±1.37***
SOS2	1.00±0.27	1.17±0.31	1.21±0.25	1.87±0.66
WRKY22	1.00±0.34	0.43±0.18	0.61±0.11	0.72±0.24
WRKY25	1.00±0.26	0.93±0.27	1.68±0.37	2.32±0.44**
WRKY29	1.00±0.36	0.82±0.27	1.66±0.49	1.53±0.30
ZAT12	1.00±0.17	0.24±0.13**	0.97±0.38	3.43±0.6***
Gene expression profile of METABOLIC ENZYMES				
GAPDH	1.00±0.02	0.51±0.18*	0.38±0.12**	0.38±0.09***
GAPC	1.00±0.25	0.62±0.21*	0.58±0.18*	0.80±0.2
ADH1	1.00±0.26	0.20±0.08**	0.16±0.06**	1.13±0.96
LDH1	1.00±0.27	0.64±0.23	0.46±0.16	1.11±0.12
PDC1	1.00±0.08	0.38±0.11**	0.14±0.05**	0.79±0.48
Gene expression profile of Phenylalanine Ammonia Lyase				
PAL1	1.00±0.30	0.58±0.19	0.73±0.23	0.53±0.15
PAL2	1.00±0.32	0.80±0.34	0.62±0.17	0.41±0.17**

Table 7.2 *(page 97): Transcript level of ROS-producing and antioxidative enzymes, expressed relative to the control, in the roots of 3-week-old Arabidopsis thaliana seedlings exposed to different Cd concentrations during 24h. Each point represents the mean of five biological replicates ± SE (significance levels : * : p<0.1; ** : p<0.05; *** : p<0.01).*

7.2.2.2.2 Transcript profiles of genes related to the Ca^{2+} signal transduction pathway

Not only the MAPK-pathway was transcriptionally altered, but differences were also noticed in the transcript profiles of other regulatory pathways. Most of the Ca^{2+}- , calcineurin and calmodulin - related genes, such as Ca^{2+} (and calcineurin/calmodulin) -dependent kinases, transcription factors, transporters and other proteins, were upregulated during Cd stress (table 7.1). The gene expression of *CDPK1 (calcium dependent protein kinase 1)* as well as the gene expression levels of components of the SOS (salt overly sensitive) - pathway, were additionally determined using Real Time PCR. A significant increase in *CDPK1* expression was observed after exposure to 10 µM $CdSO_4$. The transcript level of *SOS2* was significantly increased after 5 and 10 µM $CdSO_4$.

7.2.2.3 Clustering analysis

A clustering analysis was performed to identify co-ordinately regulated genes in the roots of *Arabidopsis thaliana* after Cd exposure (figure 7.5). Based on the raw gene expression values, several MAPK as well as Ca^{2+}-regulatory genes were simultaneously induced. From this cluster, also ROS-related genes were co-ordinately regulated with specific signal transduction genes during Cd toxicity (f.e.: NADPH oxidases and MAPkinases).

7.3 Discussion

7.3.1 Cadmium induces morphological changes in the roots of Arabidopsis thaliana

Cadmium exposure leads to macroscopic damage such as growth inhibition, chlorosis and necrosis, and disrupts physiological processes such as transpiration, photosynthesis and transport. Although no significant growth reduction was noticed in the roots of *Arabidopsis thaliana* seedlings exposed to 2, 5 and 10 µM $CdSO_4$, a decrease in cell wall thickness of xylem vessels was observed (figure 7.2). The significant downregulation of *PAL1* and *PAL2* (phenylalanine ammonia-lyase) transcripts in the roots of these Cd-exposed

plants may suggest a Cd-induced inhibition of the lignin synthesis essential in cell wall formation (table 7.1). This is in contrast with other studies where an exposure to 1 µM $CdCl_2$ resulted in increases in lignin content as well as in the activity of peroxidases and laccases (important in the polymerization of monolignols to lignin) in soybean root tips (Yang *et al.* 2007).

7.3.2 Cadmium induces oxidative stress in the roots of Arabidopsis thaliana

Cadmium is known to induce oxidative stress and an important role for H_2O_2 was described previously (Romero-Puertas *et al.* 2004, Garnier *et al*. 2006). Our results confirm a Cd-related H_2O_2-production in the roots of *Arabidopsis thaliana* (figure 7.4). Superoxide radicals were also identified in these Cd-exposed roots, suggesting a Cd-induced ROS burst mediated by NADPH oxidases (figure 7.3). The occurrence of such is also supported by a simultaneous upregulation of *RBOHD* and *RBOHE* (table 7.2). These membrane proteins may function as a "sensor" to produce signalling molecules, such as H_2O_2, that in turn can activate signal transduction pathways (Romero-Puertas *et al.* 2004, Torres and Dangl 2005).

Cadmium-induced lipid peroxidation, on the other hand most likely is a secondary effect of the disturbed redox status. A significant increase in TBArm was only detected at an exposure concentration of 10 µM $CdSO_4$, although *LOX1*-expression was already upregulated at lower exposure concentrations (table 7.2). It is possible that products of LOX activities, such as oxylipins, function as signalling compounds under Cd stress.

Also mitochondria are key players in plant cell redox homeostasis; multiple studies already demonstrated the ROS-producing function of these organelles in various stress situations (Noctor *et al.* 2007, Rhoads and Subbaiah 2007). Differences in gene expression patterns of mitochondrion-related enzymes such as NADH dehydrogenase, (ascorbate-dependent) oxidases/dioxygenases, AOX1A, FSD1, thioredoxines and glutaredoxines, observed in this study, also suggest a role for mitochondria during Cd stress (table 7.1). Nonetheless, as the above mentioned genes are both ROS-inducing as well as ROS-diminishing enzymes, the specific role of mitochondria in the Cd-induced ROS production remains to be revealed.

7.3.3 GSH as a key metabolite in Cd-induced antioxidative defence in the roots of Arabidopsis thaliana

The role of GSH in the detoxification and cellular defence against Cd was also confirmed in this study (figure 7.1, tables 7.1 and 2). GSH is a key component in Cd chelation as it is a precursor for the synthesis of phytochelatins. Because of the strong upregulation of *GSH1*, combined with an induction of *PCS1 (phytochelatin synthetase 1)*, an increase in phytochelatin synthesis is suspected in the roots of *Arabidopsis thaliana* plants exposed to different Cd concentrations (table 7.1+2). *GSH2* appeared to be inhibited in the roots after 24h exposure to 2, 5 and 10 µM of $CdSO_4$, but other research confirmed a transcriptional upregulation of this enzyme after longer exposure times (Semane *et al.* 2007).
GSH also exerts its defence role via other mechanisms and serves as a co-substrate for antioxidative enzymes such as glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-S-transferases (GST) during heavy metal stress (figure 7.1) (Xiang and Oliver 1998, Dixon *et al.* 2002, Craciun *et al.* 2006). After binding with toxic compounds, GSH-complexes can be transported to specific cellular compartments such as the vacuole (Sanchez-Fernandez *et al.* 2001, Klein *et al.* 2006). As expected, various glutathione-related enzymes and/or transporters differed significantly in their gene expression patterns during Cd toxicity (tables 7.1 and 2).

7.3.4 Cadmium-induced ROS trigger defence mechanisms and signal transduction cascades in the roots of Arabidopsis thaliana

ROS are able to induce antioxidative defence mechanisms directly, for example via the "antioxidant responsive element" (ARE) commonly found in the promoter region of such genes (Scandalios 2005). One of these ARE-induced genes is *CAT1*, which gene expression was significantly upregulated during Cd toxicity (table 7.2). Cytosolic ascorbate peroxidases (APX) such as APX1 and APX2, and the plastidic iron superoxide dismutase (FSD1) are also known to be activated by H_2O_2 in various stress situations (Volkov *et al.* 2006), and the pivotal role of H_2O_2 as a key signalling compound was again underlined by the results presented in this study (tables 7.1 and 2). Especially FSD1 and APX2 seem central factors in the Cd-induced defence in the roots of *Arabidopsis thaliana* plants.

Secondly, ROS can activate scavenging mechanisms via redox-sensitive transcription factors or via the activation of kinase cascades which in turn activate transcription factors that trigger target gene transcription (figure 7.1) (Mittler *et al.* 2004, Scandalios 2005). The MAPK (mitogen activated protein kinases) pathway has been mentioned before as a rapid activation mechanism after Cd application (Jonak *et al.* 2004). Apparently, Cd also triggers *de novo* protein synthesis of these components, as our results show an upregulation of MAPKKKs (*MEKK1-ANP1-ANP2-OXI1*), MAPKKs (*MKK2)* and MAPKs (*MPK3-MPK4*) (figure 7.1, table 7.2). Transcription factors such as HSF, ZAT, WRKY and MYB (among which several that are differentially expressed under Cd) can be activated via multiple components of the MAPK cascade (figure 7.1, tables 7.1 and 2) (Kovtun *et al.* 2000, Mittler *et al.* 2004). A possible role of WRKY25 and ZAT12 in the activation of antioxidative defence mechanisms is suspected during Cd toxicity (table 7.2). ZAT12 is already known as a positive regulator of the *APX1* and *WRKY25* transcription and ZAT12, WRKY and MPK3 are all positively regulated by H_2O_2 (figure 7.1, table 7.2) (Davletova *et al.* 2005, Miller and Mittler 2006).

Based on the results of this microarray expression screening, not only the MAPK cascade pathway was transcriptionally upregulated, but also various components involved in the PA (phosphatidic acid) signalling, such as PLD (phospholipase D), are important elements of the responses of *Arabidopsis thaliana* roots to Cd exposure (table 7.2). PLDs are activated by several developmental and environmental signals to produce PA, which in turn activate ROS inducing enzymes among which NADPH oxidases (figure 7.1, table 7.2) (Wang *et al.* 2006).

A third cascade important in the regulation during Cd toxicity are transduction pathways related to the Ca^{2+} signalization (table 7.1). A potential role for Ca^{2+}, H_2O_2 and their related components in the regulation of Cd-induced effects was also observed in other studies (Garnier *et al.* 2006). Several Ca^{2+}-binding sensors were shown to be involved in abiotic stress situations, more specifically the CDPK (Ca-dependent protein kinase) family and the SOS (salt overly sensitive) family (Kaur and Gupta 2005). A specific role of the SOS-pathway has been implicated in ion homeostasis during salt stress (Serrano and Rodriguez-Navarro 2001). Apparently, components of this pathway are also induced by

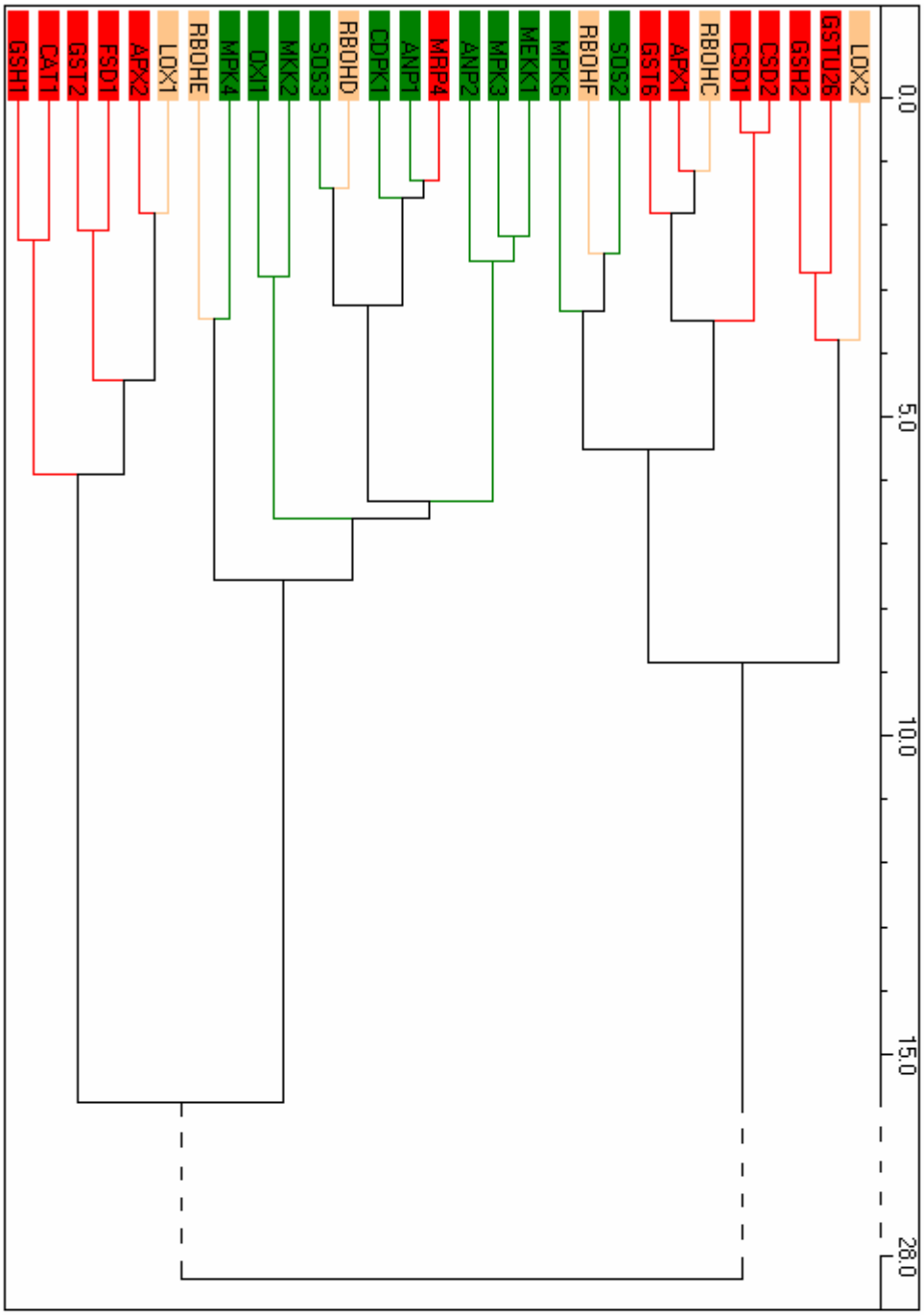
other stress situations, since this study showed a transcriptional upregulation of *SOS3* (CBL4) and *SOS2* in roots after Cd exposure of *Arabidopsis thaliana* seedlings (table 7.2). The gene expression of another Ca^{2+}-binding protein, CDPK1, was also highly upregulated in the roots during Cd stress (table 7.2). CDPKs are implicated as important sensors of Ca^{2+} fluxes in plants (Ludwig *et al.* 2004).

In summary, the roots of *Arabidopsis thaliana* seedlings showed a strong upregulation of antioxidative enzymes (APX2, FSD1, GST2) at almost all treatments, whereas NADPH oxidases and signalling components were significantly induced at the highest exposure concentration. A rapid activation mechanism, such as H_2O_2 (whether or not via a rapid MAPK phosphorylation on the protein level), is suspected to be responsible for this first defence activation. Secondly, a *de novo* synthesis of MAPKs and Ca^{2+}-dependent kinases exists that can lead, via the induction of specific transcription factors, to a prolonged antioxidative response.

7.3.5 Cross talk between Cd-induced responses

Cross talk between signalization pathways is common within various types of (a)biotic stress situations (Kaur and Gupta 2005). Therefore, it is important to emphasize the activation of multiple signal transduction pathways, and their coordinated regulation during Cd stress. A clustering analysis of the Cd-induced gene expression patterns showed a highly synchronized expression of interacting components of the MAPK pathway (*MEKK1*/*MPK3-ANP2* and *OXI1/MKK2*) (figure 7.5). As MAPKs are known to act as a rapid response mechanism, it is possible that a positive (self-maintaining) amplification loop exists within this signalling network (figure 7.1). *ANP1*, on the other hand, was simultaneously expressed with *CDPK1*, a Ca^{2+}-binding kinase, which confirms the recently demonstrated cross talk between CDPKs and MAPKs under Cd stress (figure 7.1) (Yeh *et al.* 2007).

Figure 7.5 *(page 103): The gene expression patterns of the measured data were analyzed and grouped by means of hierarchical clustering, performed with the GenEx software version 3.4. Genes that form a cluster have similar expression profiles. The analysis was based on the "Unweighted pairs linkage", which defines the distance between groups (treatments) as the average of the distances between all pairs of individuals in the two groups. The distances are calculated based on the Euclidian Distance Measure. (ROS-producing components = pink/ antioxidative components = red/ signalling components = green)*


GSH1
CAT1
GST2
FSD1
APX2
LOX1
RBOHE
MPK4
OXI1
MKK2
SOS3
RBOHD
CDPK1
ANP1
MAP4
ANP2
MPK3
MEKK1
MPK6
RBOHF
SOS2
GST6
APX1
RBOHC
CSD1
CSD2
GSH2
GSTU26
LOX2
0.0
5.0
10.0
15.0
28.0

Secondly, in the present study a transcriptional co-regulation of these signalling components with ROS-inducing genes, such as NADPH oxidases, was also observed (figures 7.1 and 5). As NADPH oxidases are involved in the Cd-induced MAPK activation (Yeh *et al.* 2007), a coordinated transcriptional regulation of these enzymes, such as RBOHE and MPK4 or RBOHF and MPK6, is not unexpected. Given the cross talk between MAPK and Ca-dependent kinases together with the coordinated regulation between *RBOHD* and *SOS3*, it is possible that Ca^{2+}-dependent enzymes and NADPH oxidases are operating together in a Cd-induced regulation response (figure 7.1).

7.3.6 Conclusion

In conclusion, during Cd stress a synchronized regulation response of different signalization cascades was detected at the transcriptional level. Moreover, the transcription of ROS-inducing enzymes seems coordinately regulated with the transcription of several of these signalling components. Taken together, these findings suggest that the cooperation of both NADPH oxidases and signalling enzymes is essential in the activation of an appropriate defence response towards Cd stress, and a causal role for ROS as a common regulation mechanism is suspected.

Chapter 8

Reactive oxygen species contribute to the induction of signal transduction pathways in the leaves of *Arabidopsis thaliana* during cadmium exposure

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Abstract

In the present study, the ROS (reactive oxygen species) profile, ROS-related mechanisms and regulation pathways were analysed in the leaves of Cd-exposed *Arabidopsis thaliana* plants. The ROS signature in the leaves was defined by a significant increase in both H_2O_2 and $^{\circ}OH$ contents after Cd exposure. The most important transcriptional changes in the leaves were related to signal transduction cascades. An induction of H_2O_2, via NADPH oxidases or directly from the roots, is suggested as an important regulation mechanism during Cd toxicity, with OXI1 as a key component in this signal transduction cascade. In addition, clustering analysis revealed an organized cooperation between ROS-inducing, antioxidative and regulatory mechanisms as a cause and effect of a Cd- (and organ-) characteristic oxidative stress signature.

8.1 Introduction

Cadmium induces oxidative stress in plants almost immediately after the start of exposure. Especially H_2O_2 plays a key role in this Cd-induced cellular redox disequilibrium (Garnier *et al.* 2006, Rodriguez-Serrano *et al.* 2006). H_2O_2 is most likely produced via an increased activity of NADPH oxidases, although mechanisms such as ROS production via mitochondria as well as an inhibition of the antioxidative defence system are also described during Cd stress (Romero-Puertas *et al.* 2004, Drazkiewicz *et al.* 2007, Yeh *et al.* 2007). ROS, such as H_2O_2, are ideal signalling molecules and they can influence the expression of a number of genes involved in processes such as signal transduction and cell rescue (Desikan *et al.* 2001, Gadjev *et al.* 2006). The induction of lipid peroxidation via lipoxygenases is another feature of Cd-induced oxidative stress (Semane *et al.* 2007, Smeets *et al. in press*). Lipoxygenases are a family of enzymes that catalyze the oxygenation of polyunsaturated fatty acids involved in responses to stresses among which Cd and Cu (Skorzynska-Polit *et al.* 2006).

Organisms try to counteract the induced oxidative stress condition by activating several components of their antioxidative defence system. This defence activation can be (post)translational, or as recently shown transcriptional (chapter 4). Since oxidative stress is a common effect induced by multiple stress factors, several common hypotheses exist behind this antioxidative reaction capacity. ROS can be detected by mechanisms such as ROS receptors, redox-sensitive transcription factors and inhibition of phosphatases (Mittler *et al.* 2004). In this way, signal transduction cascades are activated, either directly or via secondary signalling molecules such as Ca^{2+} (Bhattacharjee 2005). Both MAPKs (mitogen-activated protein kinases) as well as Ca^{2+}-dependent kinases are described as important mediators during multiple stress situations. Nevertheless, differences in stress and regulation parameters depend on the specificity of the stress factor, which makes it highly important to unravel the complexity of these mechanisms in multiple stress conditions.

During Cd stress, changes in the protein and/or activity level of MAPK components are described (Jonak *et al.* 2004, Yeh *et al.* 2007). A coordinated link between Cd exposure and Ca^{2+} signalling was also demonstrated, whether or not via H_2O_2 as an intermediate signalling molecule or as a second messenger

produced via a Ca^{2+}-induced oxidative burst (Garnier *et al.* 2006). Because of their similarities in structure, Cd can enter cells via Ca^{2+} channels and possibly disturbs Ca^{2+}-related processes (Perfus-Barbeoch *et al.* 2002). In previous chapter, we demonstrated the importance of both MAPK and Ca^{2+}-dependent kinases during Cd stress in the roots of *Arabidosis thaliana.* A coordinated regulation response of these signalization cascades was detected at the transcriptional level and a role for ROS as a common regulation mechanism and a missing link between the ROS-related enzymes and the signalling cascades was described.

In order to gain more insight in the molecular mechanisms behind Cd stress, the ROS profile, ROS-related mechanisms and regulation pathways were analysed in the leaves of Cd-exposed *Arabidopsis thaliana* plants. As differences in antioxidative patterns between roots and leaves were previously described at the same Cd concentrations (Smeets *et al. in press*), it is of great importance to expand this knowledge with more information regarding to the regulation mechanisms. Differences in gene regulation profiles were highlighted in this study by means of a clustering analysis.

8.2 Results

In the present study, no growth reduction nor severe morphological effects were observed in *Arabidopsis thaliana* plants after 24h of exposure to either Cd concentration. Like with the Cd-exposed roots (chapter 7), significant inhibitions in *PAL* (phenylalanine ammonia-lyase) transcription were observed in the leaves of the Cd-treated plants (table 8.1).

8.2.1 The cellular redox status in Arabidopsis thaliana is affected after application of Cd

8.2.1.1 ROS production

The occurrence of $^{\circ}OH$ was investigated by measuring the existence of free radicals in leaf extracts prepared in the presence of a spin trap (figure 8.1). EPR absorption was missing from control leaves. Hydroxyl radical adducts were observed at both Cd concentrations, albeit to a smaller extent in the leaves of the plants exposed to 5 µM $CdSO_4$. H_2O_2 levels were significantly increased after exposure to 5 and 10 µM $CdSO_4$ (figure 8.2). They were elevated with a factor

2-2.5 in the leaves of the Cd-exposed plants when compared to control plants. The ROS levels did not cause significant lipid peroxidation that was determined by measuring thiobarbituric acid reactive metabolites (chapter 5, figure 5.1).

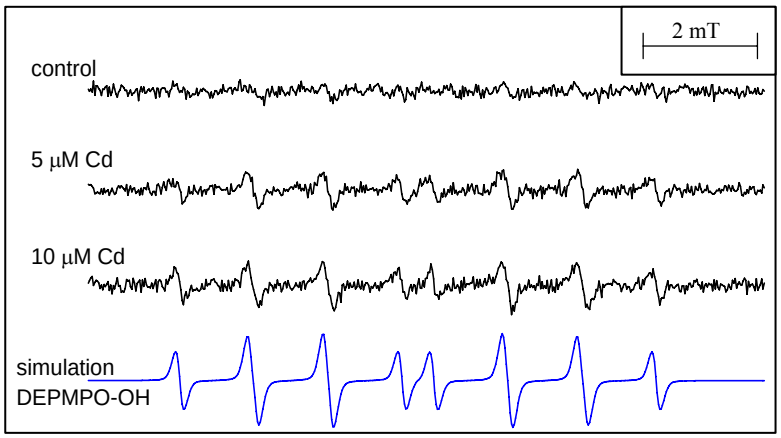


Figure 8.1: *EPR-spectra of DEPMPO-adducts produced in extracts of Arabidopsis thaliana roots exposed to 0 µM $CdSO_4$, 5 µM $CdSO_4$ and 10 µM $CdSO_4$.*

Gene expression, using Real Time PCR, was performed to determine possible sources of this ROS production (table 8.1). *RBOHC*, *RBOHE* and *RBOHF* (respiratory burst oxidase homolog) were transcriptionally induced in the leaves of the Cd-exposed plants, and this upregulation was significant at all exposure levels (table 8.1). In addition, the gene expression of the cytoplasmic lipoxygenase LOX1 was increased with a factor 3 after an exposure to 10 µM $CdSO_4$, whereas the plastidic lipoxygenase LOX2 expression was already significantly upregulated from an exposure to 5 µM $CdSO_4$.

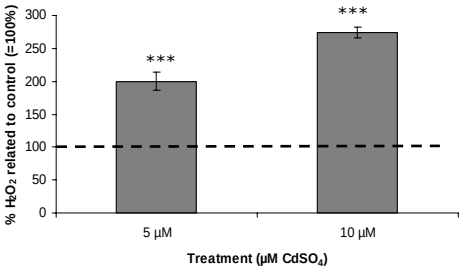


Figure 8.2*: The H_2O_2 content measured in the leaves of 3-week-old Arabidopsis thaliana seedlings exposed to 5 or 10 µM $CdSO_4$ over a 24h period or grown under control conditions (=100%). Values are mean ±S.E. of 3 biological independent replicates (significance levels: ***: p<0.01).*

Table 8.1 *(page 109): Transcript level of ROS-producing and antioxidative enzymes, expressed relative to the control, in the roots of 3-week-old Arabidopsis thaliana seedlings exposed to different Cd concentrations during 24h. Each point represents the mean of five biological replicates ± SE (significance levels : * : p<0.1; ** : p<0.05; *** : p<0.01).*

Gene	2 µM Cd	5 µM Cd	10 µM Cd
Gene expression profile of ROS PRODUCING ENZYMES			
LOX1	0.85±0.10	1.39±0.19	3.12±0.43**
LOX2	1.38±0.19	3.12±0.64*	3.65±0.58***
RBOHC	4.77±1.96**	28.82±3.27***	14.49±4.33**
RBOHD	1.06±0.12	1.09±0.05	1.39±0.06
RBOHE	5.99±1.86**	17.28±4.70***	20.41±3.86***
RBOHF	2.84±0.64**	5.42±1.31**	5.05±1.29***
Gene expression profile of ANTIOXIDATIVE ENZYMES			
CSD1	0.65±0.17*	1.06±0.19	0.83±0.15
CSD2	0.18±0.05**	0.09±0.01***	0.12±0.04***
FSD1	0.95±0.15	0.56±0.15**	3.12±0.43
CAT1	1.17±0.11	2.26±0.27**	3.20±0.43**
APX1	1.15±0.03	1.49±0.56	2.66±0.36**
APX2	2.63±1.01	3.09±1.65	2.29±0.90
Gene expression profile of GLUTATHION RELATED PROTEINS			
GSH1	0.73±0.07	0.73±0.14	0.85±0.18
GSH2	1.01±0.14	2.05±0.40	1.65±0.40
GST2	11.65±3.32***	34.88±6.56***	25.88±5.55***
GST6	1.39±0.16	1.02±0.18	0.52±0.11**
GSTU26	1.18±0.15	0.52±0.08	0.56±0.05
MRP4	2.56±0.50**	5.15±0.90***	3.53±0.71***
Gene expression profile of ENZYMES involved in SIGNAL TRANSDUCTION			
OXI1	3.59±0.58***	13.15±2.08***	13.07±0.73***
ANP1	1.33±0.17	1.24±0.14	0.87±0.18
ANP2	1.72±0.30	3.92±0.54**	1.80±0.21**
MEKK1	1.27±0.11	1.42±0.25	1.18±0.11
MKK2	2.71±0.34***	4.89±0.85***	4.64±0.56***
MPK3	1.81±0.20**	1.35±0.12	1.11±0.52
MPK4	1.89±0.24	3.42±0.16**	2.98±0.34**
MPK6	1.70±0.15	2.35±0.24**	1.78±0.15**
CDPK1	2.90±0.24***	4.49±0.68***	3.50±0.25***
SOS3	1.40±0.15	1.40±0.39	0.99±0.13
SOS2	1.58±0.19	2.22±0.29	2.24±0.06*
WRKY22	0.63±0.16	0.24±0.06**	0.30±0.07***
WRKY25	7.21±1.61***	12.00±1.56***	8.89±0.35***
WRKY29	1.44±0.42	0.88±0.23	0.87±0.16
ZAT12	20.36±13.77	9.64±3.16**	4.98±0.81**
Gene expression profile of METABOLIC ENZYMES			
GAPDH	0.75±0.14	0.35±0.07**	0.40±0.08**
GAPC	2.03±0.30	2.99±0.70*	2.30±0.45**
ADH1	0.84±0.22	0.63±0.13*	1.31±0.22
LDH1	0.86±0.04	1.61±0.12	1.32±0.11
PDC1	0.77±0.23	0.69±0.13	1.04±0.36
Gene expression profile of Phenylalanine Ammonia Lyase			
PAL1	0.25±0.53***	0.14±0.03***	0.21±0.06***
PAL2	0.42±0.06***	0.17±0.03**	0.25±0.55***

8.2.1.2 ROS scavenging

Several components of the antioxidative defence system were measured at the transcriptional level. Significant downregulations of *CSD1* (copper zinc superoxide dismutase 1) and *CSD2* (copper zinc superoxide dismutase 2) expression were observed in leaves of plants exposed to 2 µM $CdSO_4$, whereas the *CSD2*-transcript levels were also decreased at 5 and 10 µM (table 8.1). The *FSD1* (iron superoxide dismutase 1) gene, on the other hand, was transcriptionally downregulated in the leaves of the plants exposed to 5 µM $CdSO_4$, but again upregulated at 10 µM $CdSO_4$ (table 8.1).

Concerning the H_2O_2-quenching enzymes, significant upregulation in the gene expression of the peroxisomal enzyme CAT1 was noticeable at an exposure to 5 and 10 µM $CdSO_4$. The gene expression level of *APX2* (ascorbate peroxidase 2) was slightly upregulated at all exposure concentrations, whereas the expression of the *APX1* (ascorbate peroxidase 1) gene was significantly increased in the leaves of the plants exposed to 10 µM $CdSO_4$.

Several glutathione-related genes, such as glutathione-S-transferases, glutathione transporters and glutathione-related antioxidative enzymes, differed significantly from the control plants (table 8.1). *GST2* (glutathione-S-transferase 2) and the GSH-related transporter *MRP4* (multidrug resistance protein 4) expression was strongly upregulated under all exposure concentrations, whereas no significant changes were observed in the transcriptional level of *GST26* (glutathione-S-transferase 26). The gene expression level of *GSTU6* (glutathione-S-transferase 6) was significantly downregulated after exposure to 10 µM $CdSO_4$. Transcript levels *GSH1* (gamma-glutamylcysteine synthetase) and *GSH2* (glutathione synthetase) were respectively down- and upregulated, but none of these changes was significant.

8.2.2 Signal transduction pathways affected during Cd stress

8.2.2.1 Transcript profiles of genes related to the MAPK and Ca^{2+} signal transduction pathways

In the roots increases in the transcript profiles of regulatory genes were mainly found to be significant at the highest exposure concentration (10 µM $CdSO_4$) (chapter 7, table 7.2). In the leaves, on the other hand, the gene expression levels of *CDPK1* (calcium dependent protein kinase 1)*, OXI1* (oxidative signal inducible 1), *MKK2* (MAPkinase kinase 2), *MPK3* (MAPkinase 3) were significantly

upregulated at all exposure concentrations.The transcript levels of *ANP2* (NPK1-related protein kinase 2), *MPK4* (MAPkinase 4) and *MPK6* (MAPkinase 6) were also significantly increased at 5 and 10 µM $CdSO_4$, whereas the transcript level of *SOS2* was only significantly increased after 10 µM $CdSO_4$. No significant differences were found in *ANP1* (NPK1-related protein kinase 1) and *MEKK1* (MAPkinase kinase kinase 1) expression.

Regarding to the transcription factors, a strong upregulation of both *WRKY25* and *ZAT12* was observed at all exposure concentrations. *WRKY22*, on the other hand, was transcriptionally decreased, albeit only significant at 5 and 10 µM $CdSO_4$. No significant transcriptional changes were observed in *WRKY29* expression.

8.2.3 Clustering analysis

A clustering analysis was performed to identify co-ordinately regulated genes in the leaves of *Arabidopsis thaliana* after Cd exposure (figure 8.3). Based on the raw gene expression values, several MAPK as well as Ca^{2+}-regulatory genes were simultaneously induced. From this cluster, both ROS-inducing and antioxidative genes were also co-ordinately regulated with specific signal transduction genes during Cd toxicity (e.g.: NADPH oxidases and MAPkinases).

8.3 Discussion

8.3.1 Cadmium-induced oxidative stress signature: a dynamic process

Oxidative stress is a key feature of Cd toxicity in plants (Romero-Puertas *et al.* 2004). It is therefore essential to gather more information about Cd-induced oxidative stress profiles. The present study analysed ROS production, lipid peroxidation and gene expression profiles of ROS-producing and ROS-scavenging enzymes in the leaves of 3-week-old *Arabidopsis thaliana* seedlings exposed during 24h to 2, 5 or 10 µM $CdSO_4$. The obtained results were compared with the Cd-induced profiles in the roots that were described previously (chapter 7).

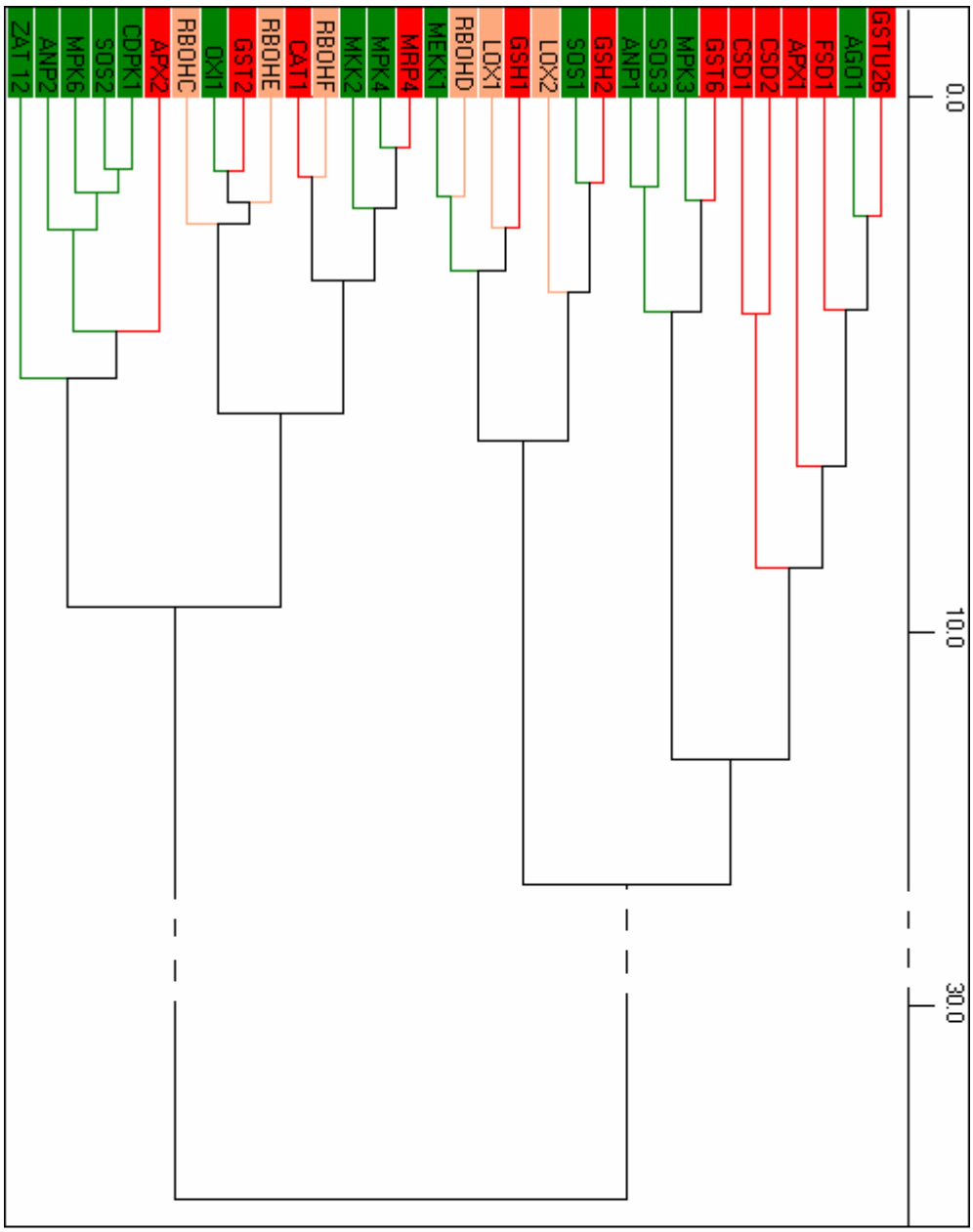


***Figure 8.3**: The gene expression patterns of the measured data were analyzed and grouped by means of hierarchical clustering, performed with the GenEx software version 3.4. Genes that form a cluster have similar expression profiles. The analysis was based on the "Unweighted pairs linkage", which defines the distance between groups (treatments) as the average of the distances between all pairs of individuals in the two groups. The distances are calculated based on the Euclidian Distance Measure. (ROS-producing components = pink/ antioxidative components = red/ signalling components = green)*

The Cd concentrations in the leaves of Cd-exposed plants were about 1/10th as compared to the roots (chapter 4), but ROS levels were nevertheless strongly increased. The ROS signature in the leaves was defined by a significant increase in both H_2O_2 en $^{\circ}OH$ contents (figures 8.1 and 2). As Cd is not a redox-active metal, ROS are likely to be induced via indirect mechanisms and an important role for both NADPH oxidases (RBOH) and lipoxygenases (LOX) was confirmed in our results (table 8.1). Secondly, Cd can replace essential elements such as Fe and Cu that can generate $^{\circ}OH$ via the Fenton reaction. A third mechanism of indirect oxidative stress induction is via the disruption of antioxidative components such as a reduced activation and downregulation of CuZnSOD observed under Cd stress (table 8.1) (Drazkiewicz *et al.* 2007, Smeets *et al. in press*).

Organisms try to counteract the induced oxidative stress by activating their antioxidative defence system. Previous studies already indicated an important role for GSH in the Cd-induced antioxidative defence and detoxification (Semane *et al.* 2007). In the leaves of the Cd-exposed plants in our study, the antioxidative role of GSH was again confirmed (table 8.1). Similar transcriptional patterns were detected in roots and leaves (table 8.1) (chapter 7), with special attention for sequestration and compartmentalization (GST2 and MRP4) in both plant organs that is essential during Cd stress (Van Belleghem *et al.* 2007). Other antioxidative defence mechanisms, localized in different cellular compartments, were also increased at the transcriptional level. Interestingly, whereas ROS-producing genes were significantly upregulated at all exposure concentrations, most of the antioxidative genes (such as *FSD1, CAT1, APX1,* …) were only induced at 5 and/or 10 µM Cd. In comparison with the roots of these Cd-exposed plants (chapter 7), the antioxidative defence was induced to a lesser extent.

The generation of oxidative stress in the leaves might also originate from root-to-shoot signalling, since the Cd concentration is much higher in roots than in leaves. Both LOXes and RBOHes are suggested as possible mediators of these Cd-induced processes via the production of respectively jasmonates and H_2O_2 (table 8.1) (Maksymiec 2007). Due to the interorgan signalling, ROS production in the leaves can occur as mentioned earlier, but also the site of ROS production is of major importance and differs from roots. When Cd itself or signalling

hormones (e.g. jasmonates) arrive in the leaves, membrane molecules such as NADPH oxidases may function as a primary 'sensor' to produce secondary signalling molecules, such as H_2O_2, that in turn can activate signal transduction pathways and/or induce antioxidative defence mechanisms (Scandalios 2005, Torres and Dangl 2005). Besides these membrane-bound enzymes, strong evidence exists towards both mitochondria and chloroplasts as important sources of ROS-production (Noctor *et al.* 2007). Changes in plastidic (*CSD2, FSD1*-expression) as well as in mitochondrial (*CAT1* expression) defence systems were detected in our study (table 8.1). Several of the plastidic antioxidative components are operating under the influence of *APX1* (Davletova *et al.* 2005), which is also significantly upregulated in the leaves after Cd exposure (table 8.1). The mitochondrial enzyme GAPDH, showing a trend of downregulation (table 8.1), is inactivated by H_2O_2 *in vitro*, potentially placing it in a signalling cascade induced by ROS (Hancock *et al.* 2005).

It can be concluded that most of the ROS-producing and antioxidative enzymes induced by Cd seem important in both roots and leaves (chapter 7). Differences however appear to be organ-specific, such as the ROS signature (H_2O_2 and $O_2^{\circ-}$ in the roots (chapter 7) and H_2O_2 and $^{\circ}OH$ in the leaves), and the expression profiles of ROS producing and ROS scavenging enzymes. The specificity of these stress mechanisms is probably dependent of differences in regulation mechanisms (cfr. *infra*) and the place of ROS production, which can be influenced by the metal concentration, the metal speciation and time of exposure.

8.3.2 Cadmium-induced ROS and signal transduction cascades

H_2O_2 is a common signal in both plant organs under Cd toxicity (cfr. *supra*). A detailed regulation behind this H_2O_2 induction, however, is still not clear, but a central role for NADPH oxidases was demonstrated in our results. Both up- and downstream of the H_2O_2-specific signal, little is known about the signalling cascades that induce or activate specific ROS-producing and scavenging mechanisms during Cd stress. Multiple studies demonstrated a primary role of MAPKs in Cd-induced signalling in all kinds of organisms (Kefaloyianni *et al.* 2005, Lag *et al.* 2005, Zhang *et al.* 2006). In plants, the role of MAPK during Cd stress was mainly examined at the metabolic level in roots (Jonak *et al.* 2004,

Yeh *et al.* 2007), or in cell cultures. Complementary to these results, a transcriptional analysis of enzymes involved in signal transduction was performed in the leaves of *Arabidopsis thaliana* exposed to 2, 5 and 10 µM $CdSO_4$ during 24h (table 8.1).

OXI1 is known to be activated by H_2O_2 and the strong transcriptional upregulation of this enzyme at all exposure concentrations (table 8.1) demonstrates its central and primary role in the oxidative stress signalling. It is possible that under Cd stress, a common signal induces *OXI1* expression, which in its turn can activate both MPK3 and MPK6 on the one hand and specific antioxidative defence mechanisms on the other hand (table 8.1) (Rentel *et al.* 2004). Other components of the MAPK cascade were also differentially expressed in the leaves of the Cd-exposed plants. Our results show an upregulation of MAPKKKs (*ANP2-OXI1*), MAPKKs (*MKK2*) and MAPKs (*MPK3-MPK4-MPK6*), and also the gene expression level of the Ca^{2+}-dependent kinase CDPK1 was significantly upregulated (table 8.1). The induced kinase cascades can activate transcription factors as is presumably the case in the leaves of Cd-exposed plants where both WRKY25 and ZAT12 are highly expressed at all exposure concentrations (table 8.1). It is also possible that *WRKY25* and *ZAT12* expression, or the upregulation of antioxidative components, are regulated by the same mechanism that induces MAPK expression.

In contrast with the antioxidative upregulation, the signalling cascades were induced under all exposure concentrations (table 8.1). Furthermore, in the roots of Cd-exposed *Arabidopsis thaliana* plants, significant upregulations of both MAPkinases and Ca^{2+}-dependent kinases were only observed at the highest exposure concentration (chapter 7). We suspect that when Cd, or a root-to-shoot signalling molecule, arrives in the leaves the oxidative signalling network is triggered.

In summary, after 24h of exposure to Cd, the most important changes in the leaves were related to signal transduction cascades. An induction of H_2O_2, via NADPH oxidases or directly from the roots, is suggested as an important regulation mechanism during Cd toxicity, with OXI1 as a key component in this signal transduction cascade. As a result, similar and/or different kinases together with their specific induction patterns might activate transcription

factors that in turn induce stress responses dependent on Cd concentration and speciation as well as on the plant organ studied.

8.3.3 Cross talk between Cd-induced responses

Cross talk between signalization pathways is common within various types of (a)biotic stress situations (Kaur and Gupta 2005). NADPH oxidase and Ca^{2+}-dependent MAPK activation were already described under Cd stress in rice roots (Yeh *et al.* 2007), whereas Ca^{2+}-dependent protein kinases can regulate NADPH oxidase activation in potato (Kobayashi *et al.* 2007). In this study, a similarly regulated transcription profile of these enzymes (CDPK, MAPKs and NADPH oxidases) was demonstrated in the leaves of Cd-exposed plants by means of a clustering analysis (figure 8.3). A concurrent induction of *OXI1* with the most important NADPH oxidases, *RBOHC* and *RBOHE* was detected. *CDPK1*, on the other hand, was equally expressed with *MPK6* and *ANP2*. On the protein level a straight cause and effect relationship was detected between NADPH oxidases, CDPKs and MAPK in Cd-exposed rice roots (Yeh *et al.* 2007). The transcription profile of these enzymes, on the contrary, was likewise for the different genes, presumably as a result of the same regulation mechanism. A clear time-related induction pattern of the cooperating components, however, still has to be established. Some studies show evidence for Ca^{2+} acting upstream from ROS (Grant *et al.* 2000), whereas other investigations demonstrate a ROS burst prior to Ca^{2+}-increases, indicating a downstream role (Rentel and Knight 2004). In the case of Cd toxicity, Garnier *et al.* (2006) demonstrated the accumulation of H_2O_2 was preceded by an increase in cytosolic Ca^{2+}, essential to activate NADPH oxidases in BY-2 cells.

Several antioxidative genes, such as *GST2*, *CAT1* and *MRP4* were also similarly upregulated with these MAPK and NADPH oxidases (figure 8.3). A coordinated response via root-to-shoot signalling might induce the antioxidative defence directly as well as through the activation of signal transduction pathways, e.g. OXI1 (figure 8.3). In the roots of Cd-exposed *Arabidopsis thaliana* plants (chapter 7), *LOX1* expression was highly upregulated at all exposure concentrations. LOXes are involved in the production of jasmonates and possibly the root-to-shoot signal is mediated via a LOX-dependent jasmonate production. Another possibility is a NADPH oxidase related H_2O_2 production, wherein H_2O_2 is

presented as a long distance signalling molecule that was already described in other stress situations (Yang *et al.* 2006).

In summary, a synchronic regulation of signalization, ROS-producing and antioxidative pathways was observed in the leaves during Cd stress. As compared to the roots, differences in clustering patterns were detected and cross talk between both organs via root-to-shoot signalling can be supposed. Both NADPH oxidases and LOXes are suggested as central mediators to activate the (root) antioxidative defence on the one hand, and (leaf) signal transduction mechanisms on the other hand.

8.3.4 Conclusion

In conclusion, our results suggest an organized cooperation between ROS-inducing, antioxidative and regulatory mechanisms as a cause and effect of a Cd (and organ-) characteristic oxidative stress signature. Both NADPH oxidases and LOXes are operating as key regulators in the activation of antioxidative responses and signal transduction cascades in respectively roots and leaves during Cd stress. A time-related order of event of all induced components, however, still has to be established in further research.

Chapter 9

Heavy metal-induced signal transduction mechanisms in *Arabidopsis thaliana*: a comparison between cadmium and copper

Abstract

At the cellular level, both Cd and Cu induce oxidative stress. ROS (reactive oxygen species) can act as signalling molecules and in a stress situation, the induced redox disequilibrium can lead to the activation of multiple signal transduction mechanisms. In the previous chapters, an induction of signalling transduction pathways, such as the MAPK (mitogen-activated protein kinase) cascade, was demonstrated in both roots and leaves under Cd stress. In the present study, Cu-induced signalling responses were analyzed in both roots and leaves of *Arabidopsis thaliana.* Clustering analyses were performed to unravel common and metal-specific effects on MAPK pathways.

Our data confirm a central role of the MAPK pathway under Cu stress as a significant upregulation of various MAPK components was observed. Also other signalling molecules such as Ca^{2+} and CDPKs (Ca^{2+}-dependent kinases) are required in the Cu-induced signalling. According to our results, it is important to emphasize that signal transduction cascades are not only metal-specific, but also both roots and leaves need be investigated in order to unravel the complexity of metal-induced regulation.

9.1 Introduction

The impact of soil heavy metal contamination leads to hazardous health effects in both animals and plants (Vangronsveld and Clijsters 1994, Fargasova 2001, He *et al.* 2005). The toxic effects of Cd and Cu on plants have been extensively studied and a fast inhibition of growth as well as decreases in photosynthetic activity, transpiration and respiration were described in the past (Lagriffoul *et al.* 1998, Maksymiec *et al.* 2007). Overall, the phytotoxic effects of Cu on metabolism and growth of higher plants is more severe as compared to other metals (Fargasova 2001, Wojcik and Tukiendorf 2003).

At the cellular level, both Cd and Cu induce oxidative stress, albeit via different pathways due to their chemical properties. As described earlier (chapters 4 and 6), the increased ROS (reactive oxygen species) levels under Cd toxicity are likely to be produced via indirect mechanisms such as an inhibition of the antioxidative defence, induction of lipid peroxidation and/or an increased activation of NADPH oxidases. Copper, on the other hand, is redox-active and hence able to produce ROS directly. Via the Fenton reaction, Cu can produce hydroxylradicals ($^{\circ}OH$) leading to cellular damage such as lipid peroxidation.

Oxidative stress is often referred to as a harmful process, but ROS also regulate many physiological processes in plants (Mittler *et al.* 2004). Because they are small and diffusable over short distances, ROS are ideal signalling molecules in both control and stress conditions. In a stress situation, the induced redox disequilibrium can lead to the activation of multiple signal transduction mechanisms, such as the MAPK (mitogen-activated protein kinase) cascade (Nakagami *et al.* 2005, Zhang *et al.* 2006). More specific, a central role for OXI1 in ROS sensing and signalling (via the activation of MPK3/6) was identified (Rentel *et al.* 2004). Activation of MAPK scaffolds takes place through sequential phosphorylation of its kinase components that subsequently phosphorylate other substrates among which transcription factors and protein kinases (Pitzschke and Hirt 2006). In this way, components of the ROS scavenging network can be induced or a positive amplification loop can be activated resulting in an increased ROS production via NADPH oxidases (Mittler *et al.* 2004). Information about different genes induced by the MAPK activated transcription factors, however, remains rather fragmentary. A role for MPK6 as repressor in the

regulation of pathogen-defence responsive genes was demonstrated in rice plants (Yuan *et al.* 2007) and ZAT12 presumably induces *APX1* expression during oxidative stress (Rizhsky *et al.* 2004).

An increased activation of diverse MAPKs was also demonstrated during Cd and Cu stress, although different underlying induction mechanisms were suggested (Jonak *et al.* 2004, Yeh *et al.* 2007). In order to gain more information about the exact role of MAPK during heavy metal stress, the effect of 24h exposure of Cd and Cu on the transcript level of several MAPKs was examined. Clustering analyses were performed to unravel common and metal-specific effects on MAPK pathways. These results were linked to the redox status induced by either metal. Stress factors always trigger multiple regulation pathways, therefore the role of Ca^{2+}-dependent signalling as well as the function of miRNAs under heavy metal stress were examined.

9.2 Results

In the present study, 3-week-old *Arabidopsis thaliana* seedlings were exposed to 0.5, 2 or 5 µM $CuSO_4$ during 24h and responses were compared with the Cd-induced profiles in roots and leaves as described in previous chapters. In the roots, Cu-uptake increased significantly in function of the Cu-exposure concentration (figure 9.1A), and a significant ($p<0,05$) growth reduction was observed in the roots of the plants exposed to 2 and 5 µM $CuSO_4$ (results not shown). In the leaves, Cu concentrations were also significantly higher when exposed to increased Cu concentration, albeit only with a factor 2-2.5 (figure 9.1B). No significant morphological changes were observed in the leaves of the Cu-exposed plants (results not shown).

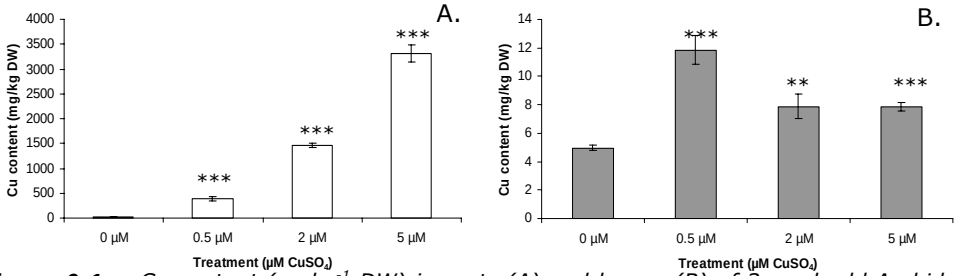


Figure 9.1*: : Cu content (mgkg^{-1} DW) in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana seedlings whether or not exposed to 0.5, 2 or 5 µM $CuSO_4$ over a 24h period. Values are mean ± S.E. of 4 biological independent replicates (significance levels: **: p<0.05; ***: p<0.01).*

As Cu is known to induce lignification, *PAL* (phenylalanine ammonia-lyase) expression was measured in both roots and leaves. A significant upregulation in *PAL* (phenylalanine ammonia-lyase) transcription was observed in both organs at the highest Cu-exposure concentrations (figure 9.2).

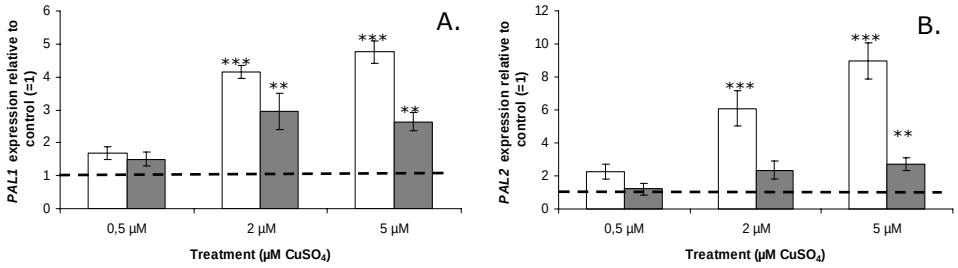


***Figure 9.2**: PAL1 (A) and PAL2 (B) expression in roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0.5, 2 or 5 µM $CuSO_4$ over a 24h period, expressed relative to the not exposed plants (=1). Values are mean ± S.E. of 5 biological independent replicates (significance levels: **: p<0.05; ***: p<0.01). PAL: phenylalanine ammonia-lyase.*

9.2.1 The cellular redox status is affected after exposure to Cu: Role of GSH

The occurrence of lipid peroxidation was investigated by measuring thiobarbituric acid reactive metabolites (TBArm) (figure 9.3). Cu did not induce lipid peroxidation in the leaves, whereas in the roots a significant increase in TBArm was detected after exposure to 2 and 5 µM $CuSO_4$.

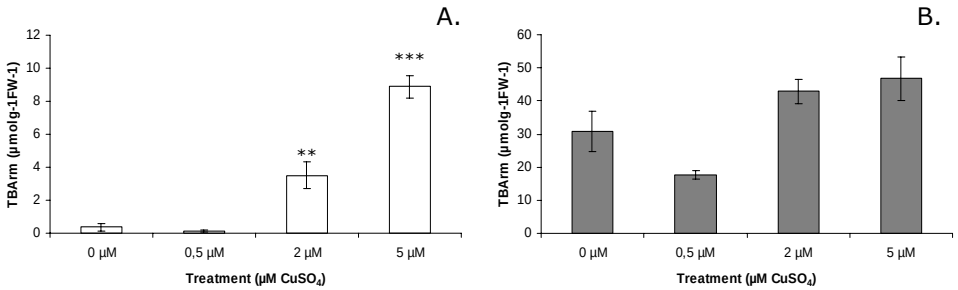


***Figure 9.3**: Lipid peroxidation measurement was based on the amount of TBA reactive metabolites and was analyzed in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana seedlings whether or not exposed to 0.5, 2 or 5 µM $CuSO_4$ over a 24h period. Values are mean ± S.E. of 5 biological independent replicates (significance levels: **: p<0.05; ***: p<0.01).*

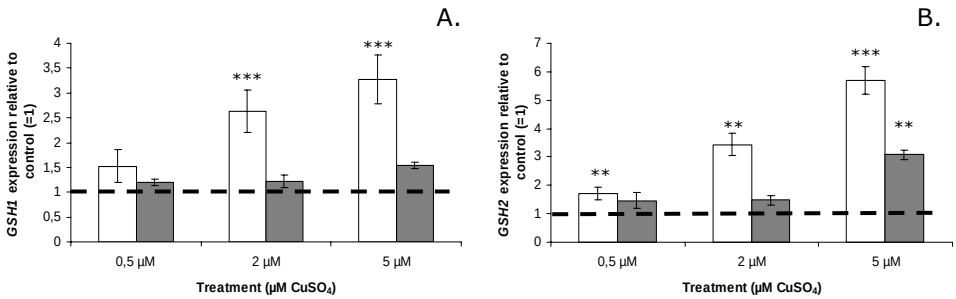


Figure 9.4*: GSH1 (A) and GSH2 (B) expression in roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0.5, 2 or 5 µM $CuSO_4$ over a 24h period, expressed relative to the control plants (=1). Values are mean ± S.E. of 5 biological independent replicates (significance levels: **: p<0.05; ***: p<0.01). GSH1: γ-glutamylcysteine synthetase; GSH2: glutathione synthetase.*

Gene expression, using Real Time PCR, was performed to determine a possible role of GSH in the Cu-induced antioxidative defence. Several glutathione enzymes involved in glutathion synthesis and glutathione-related transporters were differentially expressed as compared to the control plants. In the roots of Cu-exposed plants, transcript levels of both *GSH1* (gamma-glutamylcysteine synthetase) and *GSH2* (glutathione synthetase) were upregulated (figure 9.4). Significant induction of *GST2* (glutathione-S-transferase 2) and the transporter *MRP4* (multidrug resistance protein 4) gene expression was also noticed under all exposure concentrations (figure 9.5). No significant changes in *GST6* (glutathione-S-transferase 6) transcript level, but even a significant reduction in *GSTU26* (glutathione-S-transferase 26) expression after an exposure to 2 µM $CuSO_4$ was observed.

In the leaves of Cu-exposed plants, gene expression of GSH-related enzymes was not significantly altered, except for *GSH2* (5µM Cu) and *MRP4* (2 µM Cu) (figures 9.4 and 5).

9.2.2 Signal transduction pathways affected during Cu stress

9.2.2.1 Transcript profiles of genes related to signal transduction pathways

In the roots, gene expression levels of *CDPK1* (calcium dependent protein kinase 1), *SOS3* (salt overlay sensitive 3), of the MAPK components *OXI1* (oxidative signal inducible 1), *ANP2* (NPK1-related protein kinase 2), *MPK3* (MAPkinase 3) and the transcription factor *WRKY29* were significantly upregulated at all

exposure concentrations (table 9.1). The transcript levels of *WRKY22* and *WRKY25* were significantly increased after an exposure to 2 and 5 µM $CuSO_4$ (table 9.1).

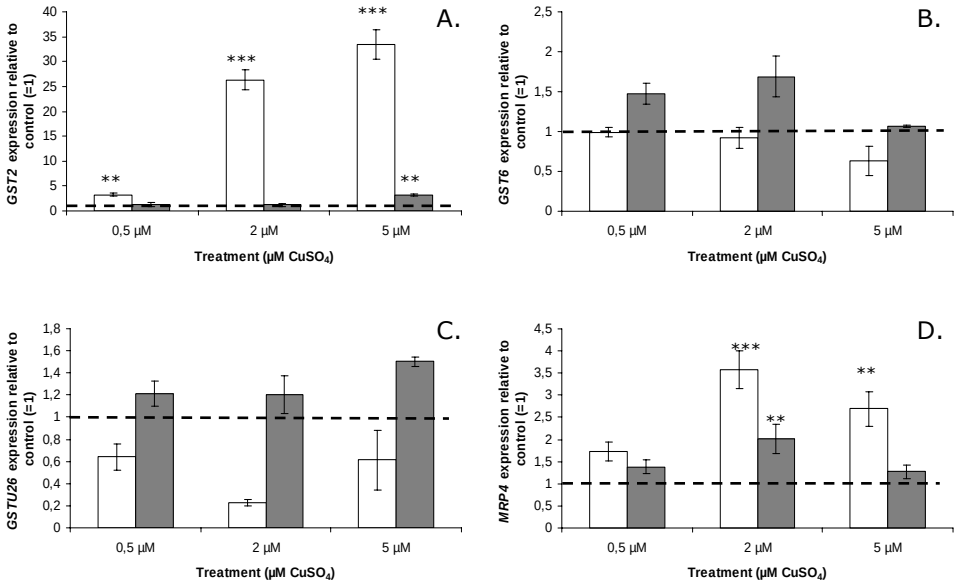


Figure 9.5*: GST2 (A), GST6 (B), GSTU26 (C) and MRP4 (D) expression in roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 0.5, 2 or 5 µM $CuSO_4$ over a 24h period, expressed relative to the control (=1). Values are mean ± S.E. of 5 biological independent replicates (significance levels: **: p<0.05; ***: p<0.01). GST: glutathione-S-transferase; MRP: multidrug resistance-associated protein.*

In the leaves, on the other hand, increases in the transcript profiles of regulatory genes were mainly found to be significant at the highest exposure concentration (5 µM $CuSO_4$) (table 9.1).

9.2.2.2 Clustering analysis

A clustering analysis was performed to identify coordinately regulated genes after both Cd and Cu exposure in the roots and leaves of *Arabidopsis thaliana* (figures 9.6 and 7). Based on the raw gene expression values, gene clusters were used to reveal metal-specific effects related to the MAPK pathway. From these clusters, similar regulated expression profiles were detected for *MPK3*/*WRKY22*, and *MPK4*/*MKK2* in both roots and leaves during Cu-stress. *WRKY25* was simultaneously induced with *OXI1/WRKY29* and *MEKK1* in respectively roots and leaves. Cd-specific clustering effects in both roots and

leaves comprehend the similar regulation patterns of *ANP1*/*WRKY22* and of *ANP2/MEKK1/MPK3*. A similar gene cluster of *OXI1*/*WRKY25* was detected in the leaves of Cd-exposed plants as compared to the roots of the Cu-exposed plants.

Table 9.1*: Transcript levels of signal transduction proteins, expressed relative to the untreated genotype (=1), in the roots and leaves of 3-week-old Arabidopsis thaliana seedlings exposed to 0.5, 2 or 5 µM $CuSO_4$ over a 24h period. Values are mean ±S.E. of 5 biological independent replicates (significance levels: *: p<0.1; **: p<0.05; ***: p<0.01). ANP: NPK-related protein kinase; CDPK: Ca^{2+}-dependent protein kinase; MEKK: MAPkinase kinase kinase; MKK: MAPkinase kinase; MPK: MAPkinase; OXI1: oxidative signal-inducible kinase1; SOS: salt overlay sensitive protein; WRKY: DNA-binding protein (transcription factor).*

	0.5 µM Cu		2 µM Cu		5 µM Cu	
Gene	ROOTS	LEAVES	ROOTS	LEAVES	ROOTS	LEAVES
OXI1	2.62±0.51**	1.16±0.33	3.87±0.67**	0.84±0.25	7.58±0.89***	2.47±1.42
ANP1	1.29±0.17	1.43±0.48	2.39±0.51	0.69±0.05**	0.99±0.09	0.89±0.09
ANP2	1.98±0.21**	1.83±0.33	5.20±3.36	1.38±0.44	2.36±0.37**	2.65±0.77
MEKK1	1.33±0.19	1.59±0.23	1.60±0.26	3.29±0.85**	4.58±3.11	1.72±0.12***
MKK2	1.30±0.04	0.96±0.21	1.95±0.72	0.79±0.15	1.29±0.12	1.18±0.20
MPK3	2.58±0.67**	1.34±0.05	2.17±0.14**	1.24±0.11	3.64±1.11**	1.35±0.12
MPK4	1.09±0.76	0.90±0.09	1.72±0.52	0.72±0.09	1.41±0.28	0.88±0.03
MPK6	1.06±0.16	1.06±0.16	1.41±0.06	1.41±0.06	1.55±0.28	1.55±0.28
CDPK1	2.18±0.15*	1.23±0.13	3.56±0.48**	1.47±0.17	5.52±1.72**	1.58±0.07***
SOS3	2.50±0.24***	1.40±0.07*	3.66±0.35***	1.46±0.28	1.56±0.10**	2.57±0.34**
SOS2	1.32±0.08	0.88±0.12	1.44±0.13	0.85±0.17	1.93±0.15	1.35±0.20
WRKY22	1.28±0.20	2.65±0.14	1.71±0.29*	2.43±0.42	2.93±0.46**	1.76±0.25
WRKY25	2.11±0.62	1.25±0.16	2.98±0.14***	1.82±0.47	3.94±0.52***	1.91±0.13**
WRKY29	2.85±0.34***	1.63±0.07	4.84±0.26***	2.13±0.41	4.06±0.38***	2.61±0.08**

9.2.2.3 MiRNA expression profiles

The microRNA profiles of miRNA398a, miRNA398b and miRNA168a were measured in the plants exposed to 5 and 10 µM $CdSO_4$ (figure 9.8). MiRNA398a and miRNA398b regulate the transcript levels of CSDs. Strong increases of both miRNAs were noticed in both roots and leaves after an exposure to 5 and 10 µM $CdSO_4$, and a concurrent inhibition in CSD-expression was observed. Especially in the leaves of the Cu-exposed plants, the opposite effect on miRNA398 and CSD-expression were found when compared to exposed plants. MiRNA168a regulates the mRNA level of AGO1 (argonaute1), an RNA slicer that selectively

recruits miRNAs and siRNAs. No significant differences were obtained for both roots and leaves under either metal exposure.

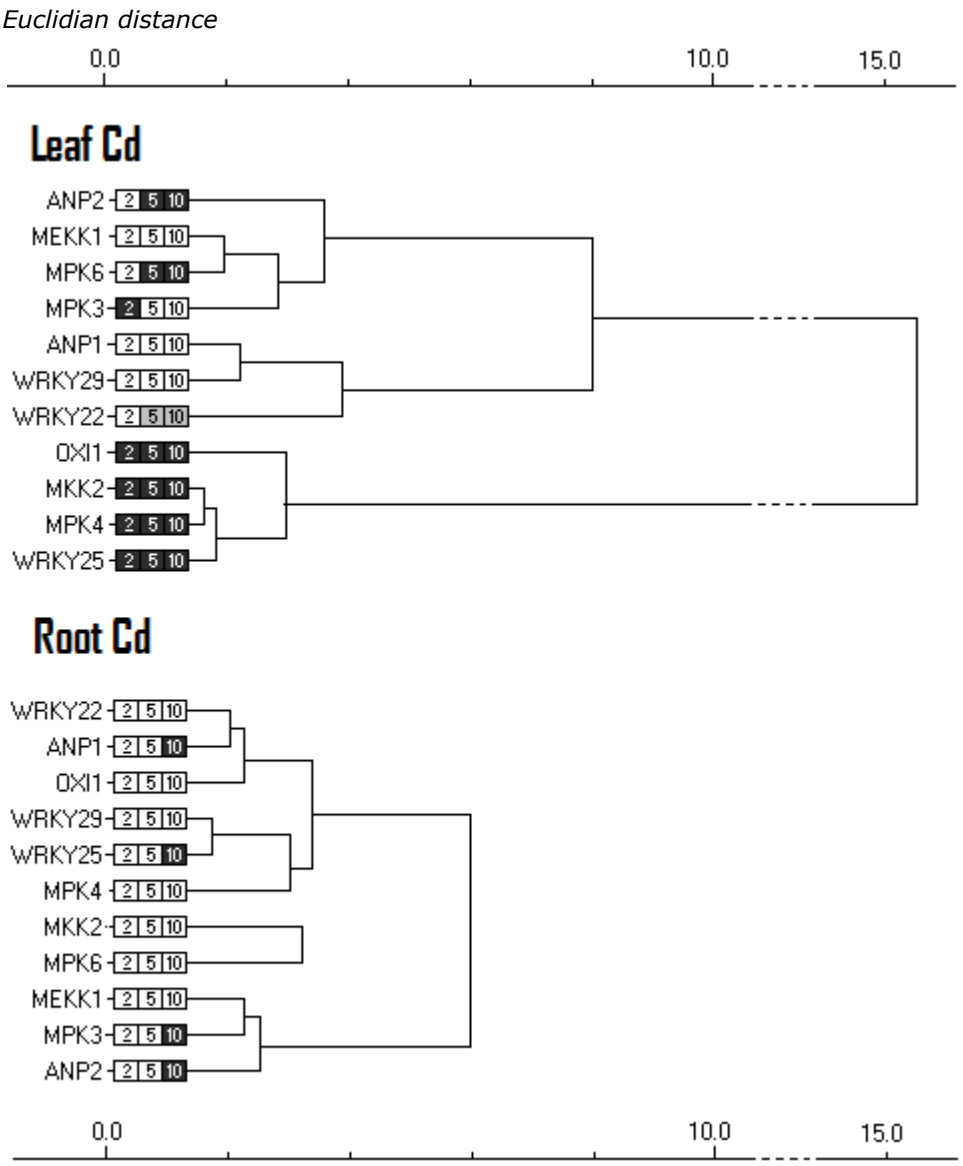


***Figure 9.6**: Cd-induced gene expression patterns of signal production proteins and transcription factors (raw data: see chapters 7 and 8) were analyzed and grouped by means of hierarchical clustering, performed with the GenEx software version 3.4. Genes that form a cluster have similar expression profiles. The analysis was based on the "Unweighted pairs linkage", which defines the distance between groups (treatments) as the average of the distances between all pairs of individuals in the two groups. The distances are calculated based on the Euclidian Distance Measure.*

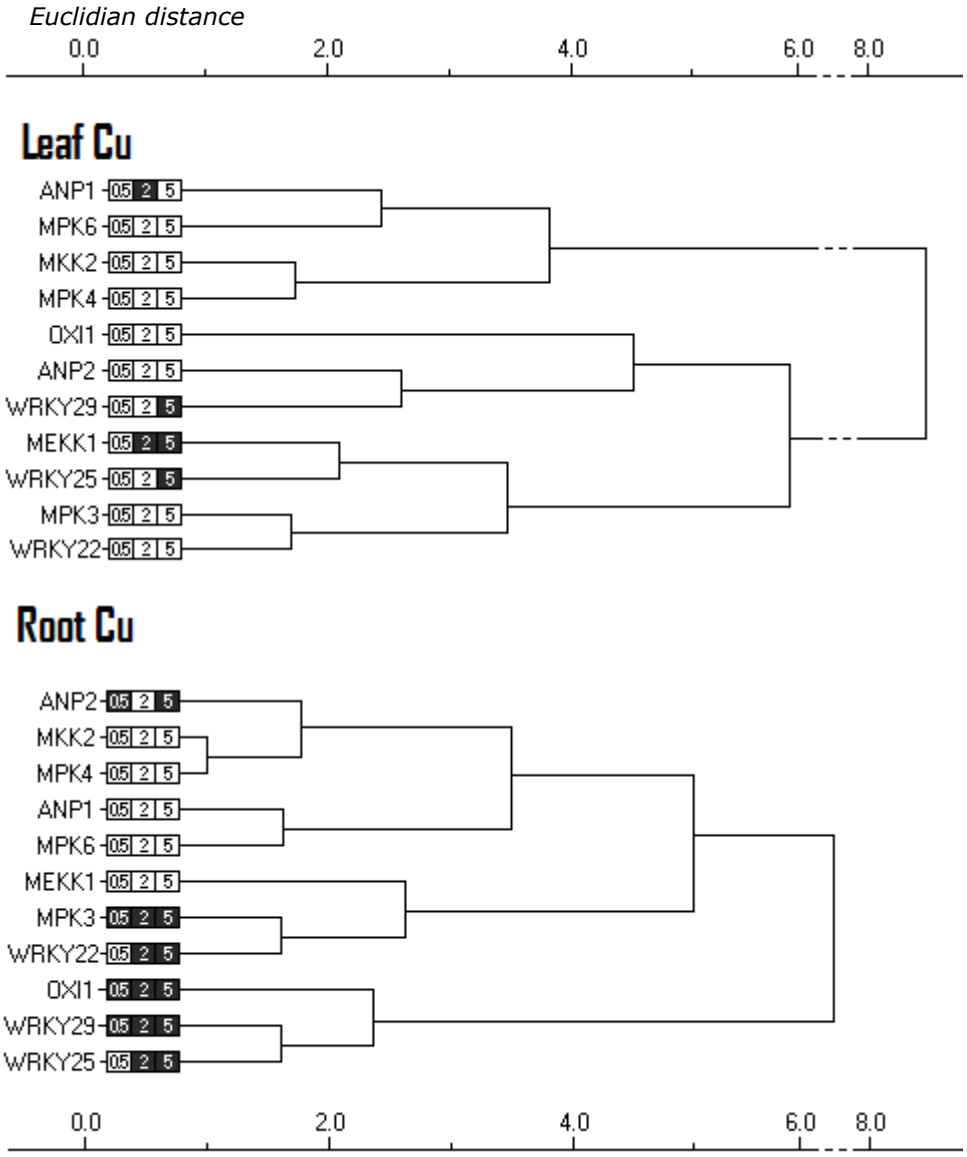


***Figure 9.7**: Cu-induced gene expression patterns of signal production proteins and transcription factors were analyzed and grouped by means of hierarchical clustering, performed with the GenEx software version 3.4. Genes that form a cluster have similar expression profiles. The analysis was based on the "Unweighted pairs linkage", which defines the distance between groups (treatments) as the average of the distances between all pairs of individuals in the two groups. The distances are calculated based on the Euclidian Distance Measure.*

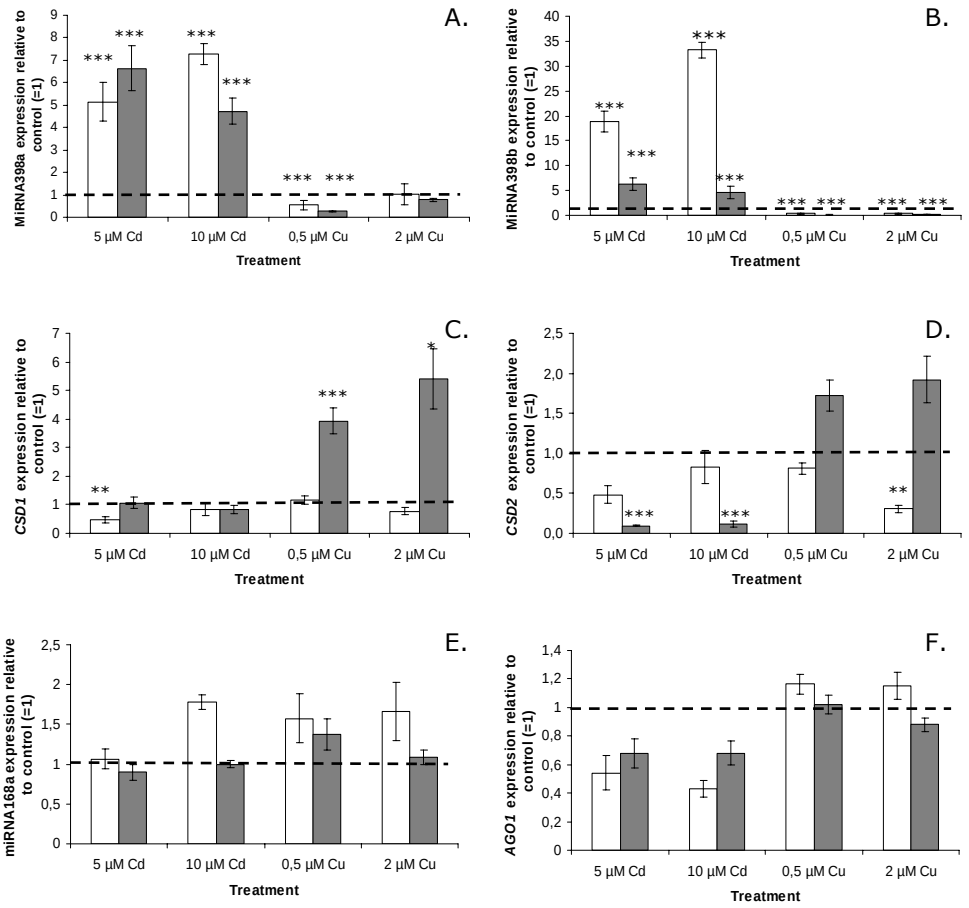


***Figure 9.8**: miRNA398a (A), miRNA398b (B), CSD1 (C), CSD2 (D), miRNA168a (E) and ARG1 (F) expression in roots (white) and leaves (grey) of 3-week-old Arabidopsis thaliana seedlings exposed to 5 or 10 µM $CdSO_4$ or 0.5 or 2 µM $CuSO_4$ over a 24h period, expressed relative to the control (=1). Values are mean ± S.E. of 5 biological independent replicates (significance levels: **: p<0.05; ***: p<0.01). AGO: argonaute; CSD: copper zinc superoxide dismutase.*

9.3 Discussion

Toxicity of Cd and Cu on plants has been extensively investigated in the past (Vangronsveld and Clijsters 1994, Cuypers *et al.* 2005, Wojcik *et al.* 2005, Clemens 2006). Although exposure to both metals results in a cellular redox disequilibrium, distinct underlying molecular mechanisms, such as ROS and its production, were demonstrated (chapters 5 and 6) (Yeh *et al.* 2007). Despite these differences, both Cd and Cu, or Cd- and Cu-induced ROS, activate MAPK signalling cascades (Jonak *et al.* 2004, Yeh *et al.* 2007).

To further improve our knowledge on cellular regulation, a transcriptional analysis of signalling components was performed under Cd and Cu stress in both roots and leaves. Three-week-old *Arabidopsis* seedlings grown on hydroponics were exposed to 0.5, 2 or 5 µM Cu during 24h. Molecular responses were compared with Cd-induced profiles in roots and leaves as described previously (chapters 7 and 8). Differences in gene regulation profiles were highlighted by means of a clustering analysis (figure 9.6 and 9.7).

9.3.1 Copper-induced oxidative stress and signal transduction cascades in the roots of A. thaliana

Copper induces oxidative stress is an indisputable statement (Cuypers *et al*. 2000, Wojcik and Tukiendorf 2003, Drazkiewicz *et al*. 2004). Due to its redox-active properties, Cu can produce ROS directly via the Fenton reaction. Organisms try to counterbalance a stress-induced redox disequilibrium by activating their defence system. The GSH (glutathione) redox state has been proven to be an important mediator of antioxidative mechanisms, such as SODs during Cu-stress (Drazkiewicz *et al.* 2007). In the present study, expression of GSH synthesis enzymes were significantly upregulated in the roots at all exposure concentrations (figure 9.4). GSH is known as a substrate in both conjugation and reduction reactions, catalyzed by glutathione-S-transferases (GSTs) that were also differentially expressed after Cu exposure (figure 9.5). An important role for GST2 in the protection against Cu-induced oxidative stress and/or as a detoxification enzyme is suspected from our results (figure 9.5). GSH-complexes can be transported to the vacuole via multi-drug resistance-associated proteins such as MRP4 (figure 9.5) (Klein *et al.* 2006). *MRP4* expression was significantly upregulated in the roots of the Cu-exposed plants and recent investigations also demonstrated a role for MRP4 in Cu-tolerance (Keinanen *et al.* 2007).

Furthermore, GSH is identified as a negative regulator of Cu-induced MAPK-activation (Yeh *et al.* 2003). It is possible that the GSH-induced repression is related to its H_2O_2-quenching properties, as H_2O_2 positively mediates the MAPK pathway (Jonak *et al.* 2004). In this study, no differences in H_2O_2 content were observed in the roots of the Cu-exposed plants (results not shown), but the produced H_2O_2 is probably immediately converted to other ROS via the Fenton-

reaction. Nevertheless, our data did confirm a central role of the MAPK pathway under Cu stress as a significant upregulation of various MAPK components was observed (table 9.1). This Cu-induced *de novo* protein synthesis implies a more long lasting function of the MAPK cascade during Cu-stress. More specific, a key role for OXI1 is suggested from these results. OXI1, and possibly also ANP2, can induce or activate the transcription factors WRKY22, WRKY25 and WRKY 29 via the activation of MPK3 (table 9.1). WRKY transcription factors are important in other stress situations such as ozone where they function as stress perception molecules via their redox-sensitive zinc-finger DNA binding domain (Tosti *et al*. 2006). Our results also suggest ROS as a common regulator mechanism, as *OXI1* and *WRKY25/29* showed similar expression profiles under Cu stress (figure 9.7).

As cross talk is related to stress-responses (Kaur and Gupta 2005), other signalling molecules such as Ca^{2+} can not be excluded as both Ca^{2+} and CDPKs (Ca^{2+}-dependent kinases) are required in the MAPK activation during Cu stress (Yeh *et al*. 2007). In this study, CDPK1, a positive regulator in controlling various stress signal transduction mechanisms (Sheen 1996), and components of the SOS-pathway were significantly upregulated as compared to the roots of the control plants. However the specificity of this underlying mechanism and an exact role of oxidative signalling in the induced MAPK-transcription during Cu stress, remains to be elucidated.

9.3.2 Copper-induced oxidative stress and signal transduction cascades in the leaves of Arabidopsis thaliana

In the leaves of Cu-exposed plants, Cu concentrations are much lower as compared to the roots (figure 9.1) (Cuypers *et al.* 2002). These results confirm earlier research where Cu accumulation was mainly detected in the root apoplast (Wojcik and Tukiendorf 2003). It is possible that an increase in lignin synthesis via PAL1 and PAL2 (phenylalanine ammonia-lyase) results in a decreased root-to-shoot translocation of Cu (figure 9.2).

Although the Cu-content in the leaves did not significantly differ between the different exposure concentrations, the transcriptional upregulation of GSH-related enzymes (figures 9.4 and 5) accompanied by a doubling in H_2O_2 content (results not shown) were only observed at the highest exposure concentrations.

This suggests the the increase of H_2O_2 and concurrent GSH production are induced via a root-to-shoot signalling mechanism at the highest exposure concentration.

In agreement with these results, a transcriptional upregulation of signalling components of both MAPK and Ca^{2+}-dependent cascades was only detected at the highest exposure concentration (table 9.1). MAPK seem not that important in the leaves of Cu-exposed plants as only *MEKK1* was upregulated. Similarities between roots and leaves were observed in the induction of specific transcription factors *(WRKY 22/25/29)*, but differences in underlying mechanisms can be expected (figure 9.7). Co-regulation of these transcription factors with signalling components in both roots and leaves was revealed by means of clustering analyses (figure 9.7). The specificity of these regulation patterns probably depends on differences in ROS content and ROS production, which can be influenced by the metal concentration, the metal speciation and underlying (root-to-shoot) signalling mechanisms.

9.3.3 Signal transduction: a comparison of Cu- and Cd-induced pathways

9.3.3.1 A differential induction of MAPkinases

The relationship between multiple signalling pathways under heavy metal stress is studied intensively. Both Cd and Cu provoke enhanced ROS production, albeit via distinct underlying pathways, each leading to MAPK activation (Yeh *et al.* 2007). Information regarding specific differences in MAPK induction, however, remains rather fragmentary. In the roots of Cd-exposed plants, a more delayed MAPK activation was detected as compared to the roots of Cu-exposed plants (Jonak *et al.* 2004). This is supported by our findings at the transcriptional level (figure 9.6 and 9.7). The upregulation of the signalling components in the Cd-exposed roots was only significant at the highest exposure concentration in contrast to Cu-exposed plants where significant increases in the MAPK gene expression level were observed at all exposure concentrations. The induction of the MAPK cascade is probably an important signal transduction event leading to appropriate cellular responses to Cu stress. Nevertheless, it has to be mentioned that *ANP2*, *MPK3* and *WRKY25* were upregulated after exposure to both metals

As both metals increase the cellular ROS content, a redox related induction of especially *WRKY25* can be expected.

In the leaves, the stress-signalling mechanism was altered and differed from roots. Whereas Cd exposure strongly increased several MAPK components at the transcriptional level, an upregulation of only one MAPK (*MEKK1*) was observed in the leaves of Cu-exposed plants (table 9.1). Similarly to the roots, the increase of expression of *WRKY25* appears to be a common metal-induced effect, although with distinct regulation profiles (table 9.1). Interestingly, *OXI1* and *WRKY25* showed similar expression patterns both in roots of Cd-exposed plants and in leaves of Cu-exposed plants. Because Cu can enhance the production of °OH (Fenton reaction) directly in the roots, and °OH content was also elevated in the leaves of Cd-exposed plants (chapter 8), this radical presumably acts as a common regulator of *WRKY25* and *OXI1* expression.

According to these results, it is important to emphasize that signal transduction cascades are not only metal-specific, but also both roots and leaves need be investigated in order to unravel the complexity of metal-induced regulation.

9.3.3.2 The role of miRNAs as signalling molecules in Cd and Cu stress

The regulation of metal-induced gene expression can also occur at the posttranscriptional level. MiRNAs function in posttranscriptional gene regulation by guiding mRNA degradation or translational repression (Sunkar and Zhu 2007). Sunkar and colleagues (2006) already showed a role of miRNA398 in the regulation of *CSDs* during Fe and Cu stress. Our data confirmed this relationship during moderate Cu stress (figure 9.8 A and B). A potential role for ROS as an underlying mechanism for the miRNA398 transcript downregulation under Cu-stress was suggested (figure 7A) (Sunkar *et al.* 2006). The opposite effect on both miRNA398 and *CSD* expression was observed under Cd-induced oxidative stress (figure 9.8 C and D). Differences in the oxidative stress signature (ROS, ROS production, place of ROS production, …) are a possible explanation for this discrepancy. We suspect that miRNA398 functions as an antioxidative defence regulator during Cu stress, whereas a role in superoxide and H_2O_2 signalling (via the regulation of *CSD1* and *CSD2* genes) is suggested during Cd stress. It is possible ROS directly regulate miRNA398 expression as no differences in the transcript level of AGO1 (argonaute1), a DICER enzyme important in the miRNA synthesis process, nor in miRNA168a, which regulates AGO1, were detected.

Specific relationships between ROS and the regulation of specific miRNAs, however, still have to be revealed.

9.3.4 Conclusion

The activation of MAPK after heavy metal-induced oxidative stress was already demonstrated in previous research. Our results confirm the MAPK induction at the transcriptional level under both Cd and Cu stress. Common metal responses but also metal-specific differences such as *WRKY22* expression and the role of miRNA398 were found. The presented data indicate that similarities and differences in regulation profiles and gene clusters can be used to detect metal-specific responses. We therefore strongly suggest to study the relation between multiple signalling components in order to unravel the diverse aspects of heavy metal toxicity.

Chapter 10

The role of the kinase OXI1 in cadmium and copper induced responses

Abstract

In the previous chapters, the importance of the MAPK pathway was demonstrated in both roots and leaves under Cd and Cu stress. The strongest induction of MAPK transcription was observed in the leaves of Cd-exposed plants and in the roots of Cu-exposed plants. In both cases, an important role for especially OXI1 in the Cd- and Cu-induced signalling was indicated by means of gene expression and clustering analyses.

In the present study, the role of OXI1 in heavy metal induced oxidative signalling was investigated after Cd and Cu exposure in both wild type and *oxi1* mutants. Our results indicate a highly important role for OXI1 in the cellular redox signalling during Cu-induced toxicity in the roots of *Arabidopsis thaliana* that was not the case for Cd stress. Cu-induced effects in the roots of these mutants influenced interorgan regulatory mechanisms and induce root-to-shoot signalling. H_2O_2 and LOXes were revealed as potential downstream targets of OXI1 under both metal stress conditions.

10.1 Introduction

The first evidence for a central role of MAPK in heavy metal toxicity came from studies with yeast or animals, where MAPKs are required in the cellular defence activation. Also in plants, both Cd and Cu activate several MAPK components at transcriptional as well as enzymatic level (Yeh *et al.* 2003, Jonak *et al.* 2004, Yeh *et al.* 2007).

Fast changes in MAPK activation and phosphorylation were described under Cu stress, whereas a more delayed activation was observed during Cd exposure (Jonak *et al.* 2004). ROS are hypothesized as key modulators of this activation and/or upregulation, although specific induction pathways are not yet revealed. Because of differences in their chemical properties, Cd and Cu probably induce MAPKs via different signalling pathways. Experiments with NADPH oxidase inhibitors suggested a major role for these enzymes in the Cd-induced MAPK activation, but also the functional state of mitochondria was implicated as an important mechanism for MAPK activation under Cd stress (Yeh *et al.* 2007). The MAPK pathway under Cu stress probably is activated after a direct Cu-induced ROS production due to its redox-active properties.

In previous chapters, the importance of the MAPK pathway was further examined in both roots and leaves under Cd and Cu stress. Gene expression analyses revealed strong differences in MAPK induction patterns that were organ and/or metal related. Furthermore although similar effects, such as induction of oxidative stress, were observed during exposure to either metal, differences in regulation profiles and gene clusters can be used to detect metal-specific responses. The strongest induction of MAPK transcription was observed in the leaves of Cd-exposed plants and in the roots of Cu-exposed plants. In both cases, one of the highest upregulated genes was *OXI1* (oxidative stress inducible kinase).

OXI1 is known to play a central role in ROS sensing during cellular processes such as root hair growth (Anthony *et al.* 2004), but also in stress situations (Rentel *et al.* 2004). Both gene expression and activity of OXI1 are strongly induced by H_2O_2 (Rentel *et al.* 2004), which makes OXI1 an essential link between oxidative burst signals and downstream responses. This kinase is also activated by phosphatidic acid (PA) in a PDK1 (phosphoinositide-dependent

kinase) related manner (Anthony *et al.* 2004). OXI1 probably functions as part of a complex network that integrates lipid and oxidative stress signals (Anthony *et al.* 2006). Downstream targets of OXI1 are likely to include MPK3 and MPK6, because H_2O_2-triggered activation of these kinases was reduced in *oxi1* mutants (Rentel *et al.* 2004).

In the present study, the role of OXI1 in heavy metal induced oxidative signalling was investigated after Cd and Cu exposure. In order to explore metal specific effects, oxidative stress related signalling responses were compared in both wild type plants and mutants lacking OXI1 (*oxi1*, Rentel, 2004).

10.2 Results

Three-week-old seedlings were exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ during 24h. Since *oxi1* mutants were available in a Ws background, growth parameters were compared between the different ecotypes of the wild type plants, *i.e.* Columbia (Col) versus Ws under both metal exposures. In agreement with previous results obtained in Col plants, no changes in growth parameters were observed in the Ws ecotype exposed to Cd (see chapter 4 and 6). In the Cu-exposed plants, however, ecotype differences were observed, as no Cu-induced growth reduction was observed in the roots of the Ws plants in contrast to the Col ecotype (see chapter 5 and 6). Previous research also indicated a higher Cu tolerance of Ws as compared to Col plants (Schiavon *et al.* 2007).

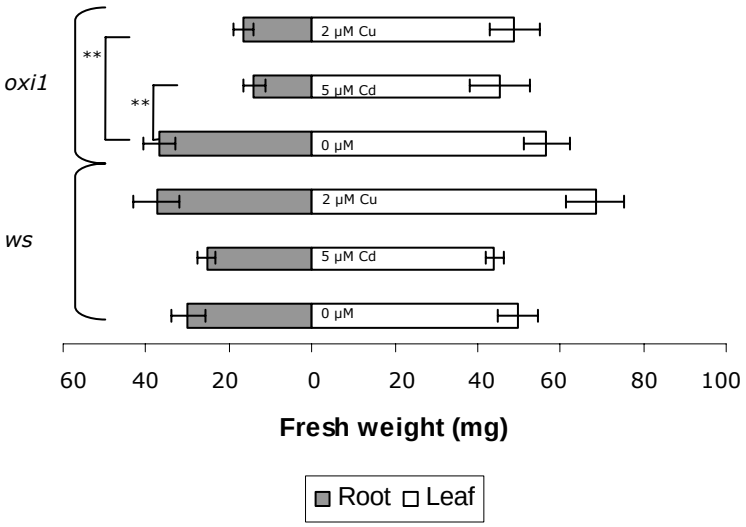


***Figure 10.1**: Leaf and root weights (mg/plant) of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ over a 24h period or grown under control conditions. Values are mean ± S.E. of ± 20 biological independent replicates (significance levels: **: p<0.05).*

10.2.1 Growth parameters and elemental profile

In the roots of wild type plants, no significant growth differences were detected between the conditions tested. In the roots of *oxi1* mutants, however, a significant growth reduction was observed in plants exposed to 5 µM Cd and 2 µM Cu as compared to the unexposed plants. In the leaves of all metal-exposed plants, no significant growth inhibition was noticed (figure 10.1).

Elemental profiles were determined, but no significant differences were observed when comparing wild type plants and *oxi1* mutants after metal exposure (results not shown). The Cd content increased significantly in both roots and leaves of all Cd-exposed plants (results not shown). Genotype differences were observed when comparing Cu-contents, as no Cu accumulated in the leaves of Cu-exposed mutants (figure 10.2B). In the roots, Cu levels increased in both genotypes, albeit to a lesser extent in the *oxi1* mutants (figure 10.2A).

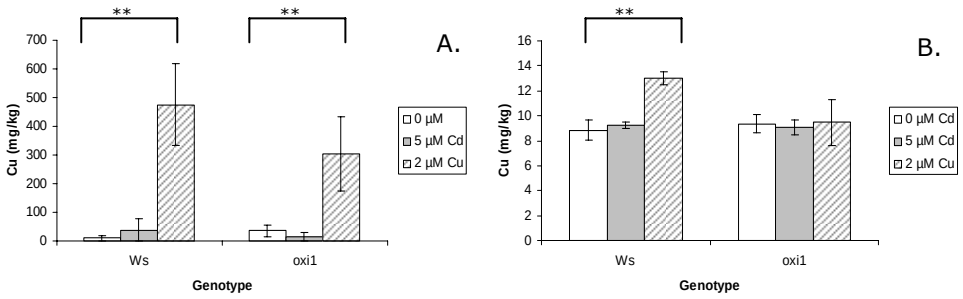


***Figure 10.2**: Cu content ($mgkg^{-1}DW^{-1}$) in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ over a 24h period or grown under control conditions. Values are mean ± S.E. of 6 biological independent replicates (significance levels: **: $p<0.05$).*

10.2.2 The cellular redox status

10.2.2.1 The level of pro-oxidants

In wild type plants, the hydrogen peroxide (H_2O_2) content increased significantly in the roots of Cd-exposed plants, whereas Cu did not influence the H_2O_2 level (figure 10.3A). In the roots of *oxi1* mutants, however, Cd did not change the H_2O_2 content, whereas an exposure to 2 µM Cu resulted in a significant H_2O_2 decrease (as compared to the unexposed *oxi1* mutants) (figure 10.3A).

In the leaves of both wild type and *oxi1* mutants, Cd significantly induced the H_2O_2-level (figure 10.3B). No significant differences were observed in the leaf

H_2O_2 content of the Cu-exposed plants, although a slight decrease was observed in the leaves of Cu-exposed *oxi1* mutants, as compared to the untreated *oxi1* mutants (figure 10.3B). Apparently, the effect of the Cu-treatment depended on the genotype, and a significant interaction effect (treatment*genotype: p=0.02) was detected.

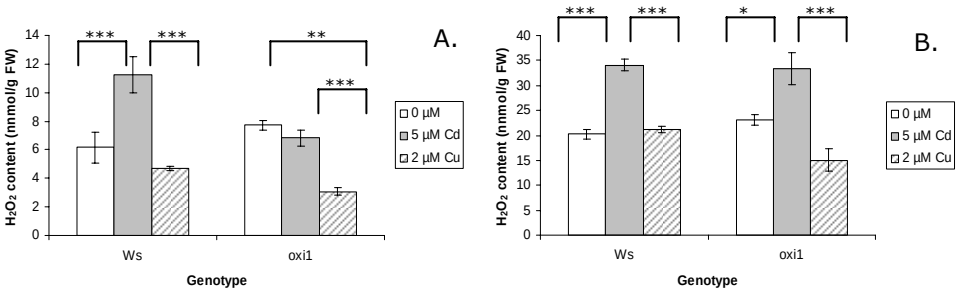


***Figure 10.3**: H_2O_2 content (nmolg^{-1} FW) in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ over a 24h period or grown under control conditions. Values are mean ±S.E. of 3 biological independent replicates (significance levels:*:p<0.1; **: p<0.05; ***:p<0.01).*

The occurrence of lipid peroxidation was investigated by measuring thiobarbituric acid reactive metabolites (TBArm) (figure 10.4). No significant differences between untreated and metal-exposed plants were observed neither in roots, nor in leaves.

10.2.2.2 Gene expression of ROS producing enzymes

In the roots of Cd-exposed plants, a significant increase in *LOX1* and *RBOHD* expression was observed in both genotypes, as compared to the untreated plants (table 10.1). Genotype differences were observed in the roots of the Cu-exposed plants, as *LOX1* transcript level was only significantly elevated in the Cu-exposed wild type plants (table 10.1). *RBOHC* transcript level was significantly downregulated by Cu in the roots of both genotypes.

In the leaves, Cd significantly induced *LOX2 and RBOHC* expression in the wild type plants, whereas in the leaves of *oxi1* mutants, this induction was diminished or even absent for *RBOHC* and *LOX2* expression respectively. *RBOHE* transcript level was stronger upregulated in the Cd-exposed *oxi1* mutants as compared to the Cd-exposed wild type plants. No significant changes were

observed in the expression level of the ROS-producing genes in the leaves of all Cu-exposed plants.

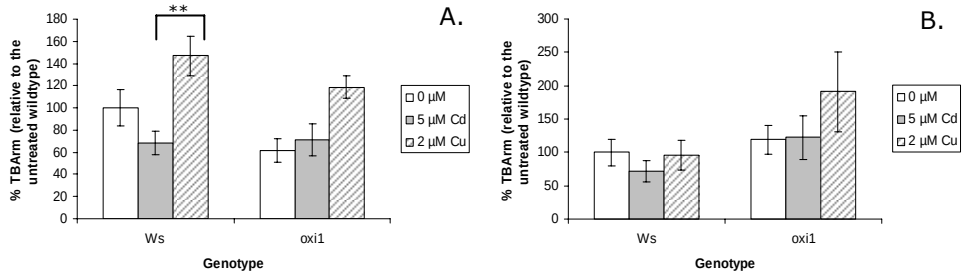


Figure 10.4*: Lipid peroxidation measurement was based on the amount of TBA reactive metabolites and was analyzed in roots (A) and leaves (B) of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CoSO_4$ over a 24h period or grown under control conditions (= 100 %). Values are mean ± S.E. of 3 (roots) or 4 (leaves) biological independent replicates (significance levels:*: p<0.1; **: p<0.05; ***: p<0.01).*

10.2.2.3 Gene expression of ROS scavenging enzymes

In the roots of Cd-exposed plants, several antioxidative genes were significantly upregulated in both genotypes. Compared to the unexposed plants, *FSD1, CAT1, APX2* and *GST2* expression were strongly induced in the roots of all Cd-exposed plants. Cu significantly reduced *CSD2, FSD1* and *APX1* transcript levels in the roots of *oxi1* mutants, although no significant inhibition effects were observed in *CSD2* and *APX1* expression in the roots of the Cu-treated wild type plants. *CAT1* transcript level, on the other hand, was significantly upregulated in the roots of Cu-exposed wild type plants but not in *oxi1* mutants.

Compared to the unexposed plants, only *GST2* was significantly upregulated in the leaves of all Cd-exposed plants. A downregulation of *CSD1/2* was observed in leaves of Cd-exposed wild type plants, whereas this was only observed for *CSD2* in Cd-exposed *oxi1* mutants. In the leaves of Cu-exposed wild type plants, *FSD1* expression was significantly downnregulated whereas the *GST2* transcript level was significantly induced. In the leaves of Cu-exposed *oxi1* mutants, a significant induction of *CSD1, CAT1* and *GST2* expression was observed.

***Table 10.1**: Transcript levels of ROS-producing enzymes, antioxidative enzymes and regulatory proteins, expressed relative to the unexposed genotype (=100%), in the roots of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ during 24h. No differences were observed in between the two unexposed genotypes (first columns). Each point represents the mean of 4 or 5 biological replicates ± SE (significance levels : downregulation: p<0,1 p<0,05 p<0,01 upregulation: p<0,1 p<0,05 p<0,01)*

ROOT	UNTREATED		CADMIUM		COPPER	
	Ws	*oxi1*	Ws	*oxi1*	Ws	*oxi1*
LOX1	1,00±0,16	1,21±0,26	1741±563	757±238	970±272	375±160
LOX2	1,00±0,27	2,57±0,47	170±75	87±49	164±17	159±60
RBOHC	1,00±0,08	0,93±0,2	88±36	108±59	21±7	33±19
RBOHD	1,00±0,24	1,5±0,19	402±181	378±165	149±17	76±23
RBOHE	1,00±0,20	1,11±0,24	187±43	250±69	79±9	46±7
RBOHF	1,00±0,19	1,14±0,20	154±49	215±77	56±7	44±16
CSD1	1,00±0,22	1,06±0,13	103±15	100±20	126±7	70±18
CSD2	1,00±0,16	1,13±0,23	85±13	116±22	84±13	40±9
FSD1	1,00±0,33	0,72±0,34	2775±667	4476±976	4±2	17±4
CAT1	1,00±0,14	1,49±0,31	1105±199	854±210	962±78	253±115
APX1	1,00±0,17	1,10±0,12	101±36	93±25	59±11	35±9
APX2	1,00±0,12	0,94±0,17	1035±308	934±186	128±38	77±12
GST2	1,00±0,21	2,12±0,81	3957±1260	1100±230	873±102	554±170
ANP1	1,00±0,09	0,96±0,08	157±31	119±18	69±11	37±8
ANP2	1,00±0,15	0,94±0,09	153±64	341±188	98±7	70±15
MEKK1	1,00±0,14	1,26±0,09	268±136	221±85	97±7	42±8
MKK2	1,00±0,15	1,17±0,11	295±53	399±122	131±20	65±13
MPK3	1,00±0,27	1,33±0,22	152±12	183±38	63±5	37±8
MPK4	1,00±0,11	1,13±0,20	224±36	170±26	66±5	36±8
MPK6	1,00±0,12	0,88±0,30	139±43	89±18	75±22	33±5
CDPK1	1,00±0,09	1,19±0,15	249±48	274±84	75±9	48±11
SOS3	1,00±0,20	1,00±0,23	375±81	559±130	142±16	79±23
SOS2	1,00±0,20	0,73±0,16	175±22	109±26	93±18	32±12
SOS1	1,00±0,20	0,73±0,16	2±1	5±4	100±66	31±6
WRKY22	1,00±0,18	1,31±0,10	50±18	40±6	37±6	17±4
WRKY25	1,00±0,16	1,38±0,14	289±28	1937±156	156±18	61±14
WRKY29	1,00±0,17	1,15±0,19	308±104	214±102	102±3	65±23
ARG1	1,00±0,18	1,07±0,06	61±17	76±13	34±1	23±5

***Table 10.2**: Transcript levels of ROS-producing enzymes, antioxidative enzymes and regulatory proteins, expressed relative to the unexposed genotype (=100%) in the leaves of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ during 24h. No differences were observed in between the two unexposed genotypes (first columns). Each point represents the mean of 4 or 5 biological replicates ± SE (significance levels : downregulation:* p<0,1 | p<0,05 | p<0,01 *upregulation:* p<0,1 | p<0,05 | p<0,01 *)*

LEAF	UNTREATED		CADMIUM		COPPER	
	Ws	*oxi1*	Ws	*oxi1*	Ws	*oxi1*
LOX1	1,00±0,18	1,24±0,33	133±35	84±6	83±22	141±19
LOX2	1,00±0,16	1,46±0,43	214±31	122±19	143±53	196±38
RBOHC	1,00±0,27	1,54±0,74	3266±1618	1815±1437	181±89	153±13
RBOHD	1,00±0,17	1,19±0,12	86±14	89±12	140±33	138±10
RBOHE	1,00±0,24	1,54±0,52	527±103	1110±349	96±25	149±17
RBOHF	1,00±0,26	0,97±0,29	185±56	92±30	60±13	202±32
CSD1	1,00±0,16	0,99±0,26	44±11	94±36	222±39	406±84
CSD2	1,00±0,20	0,85±0,28	35±6	46±17	113±13	253±65
FSD1	1,00±0,22	1,12±0,54	254±45	108±40	10±5	51±9
CAT1	1,00±0,22	1,21±0,32	155±37	132±22	88±12	177±8
APX1	1,00±0,23	0,81±0,31	115±38	274±108	83±16	189±9
APX2	1,00±0,23	0,84±0,29	157±30	124±30	135±25	224±43
GST2	1,00±0,09	1,34±0,53	3730±1273	2032±1141	236±59	469±29
ANP1	1,00±0,15	0,80±0,13	93±8	101±9	93±18	206±19
ANP2	1,00±0,19	0,80±0,19	241±89	121±43	65±15	218±26
MEKK1	1,00±0,16	0,97±0,19	152±29	154±22	92±23	144±8
MKK2	1,00±0,09	1,02±0,21	301±61	483±148	115±25	226±14
MPK3	1,00±0,14	1,06±0,16	162±27	288±53	154±33	239±29
MPK4	1,00±0,12	0,88±0,17	227±29	175±32	102±21	214±18
MPK6	1,00±0,13	0,87±0,14	104±16	110±21	109±26	231±15
CDPK1	1,00±0,21	1,25±0,36	223±75	242±60	235±144	106±9
SOS3	1,00±0,16	1,01±0,13	166±24	197±91	90±14	182±6
SOS2	1,00±0,14	0,91±0,18	109±26	49±11	91±18	155±8
SOS1	1,00±0,24	1,23±0,32	262±46	95±13	109±26	232±28
WRKY22	1,00±0,29	0,79±0,21	55±11	131±37	38±8	87±6
WRKY25	1,00±0,16	1,23±0,12	1119±281	680±267	168±37	267±52
WRKY29	1,00±0,30	0,82±0,11	84±22	91±25	65±13	148±21
NIG1	1,00±0,28	0,99±0,40	309±86	76±42	99±23	237±36
ARG1	1,00±0,21	1,09±0,19	124±13	65±10	62±14	211±18

10.2.3 Signal transduction pathways

In the roots of Cd-exposed plants, alteration of gene expression related to the MAPK pathway was limited. *MPK4* transcript level was significantly induced in the roots of Cd-exposed wild type plants, and *MKK2* expression was significantly upregulated in the roots of Cd-exposed *oxi1* mutants (table 10.1). The Ca^{2+}-dependent signalling components CDPK1 and SOS3 and the transcription factor WRKY25, however, were transcriptionally upregulated in roots of both Cd-exposed genotypes, as compared to the unexposed plants (table 10.1). The transcript level of *SOS1* and *WRKY22* was reduced in both wild type plants and oxi1 mutants, albeit only significant in the *oxi1* mutants for *WRKY22*. Copper, on the other hand, significantly reduced the transcription level of several signalling genes in the *oxi1* mutants, whereas only *WRKY22* was significantly inhibited in the roots of Cu-exposed wild type plants (table 10.1).

In the leaves, Cd significantly induced *MKK2* and *WRKY25* expression in both genotypes. *MPK4* and *SOS1* expression were only induced in the leaves of the Cd-exposed wild type plants, whereas *MPK3* transcript levels were significantly induced in the leaves of Cd-exposed *oxi1* mutants. In the leaves of Cu-exposed wild type plants, only *WRKY22* expression was significantly reduced. In the leaves of Cu-exposed *oxi1* mutants on the other hand, *ANP1/2, MPK3, SOS3, SOS1, AGO1,* and the transcription factors *WRKY25* and *N1G1* were significantly upregulated.

10.2.4 MiRNA expression profiles

In the roots of Cd-exposed plants, miRNA398b expression was significantly upregulated in both genotypes (figure 10.5 A and C). In the roots of Cu-exposed plants, miRNA398b expression was significantly downregulated in both genotypes (figure 10.5 B and D). A slight genotype difference was detected in the miRNA398a levels of both genotypes.

In the leaves, Cd significantly upregulated miRNA398a expression of both genotypes (figure 10.6A). MiRNA398b level, on the other hand, was only significantly induced in the leaves of Cd-exposed oxi1 mutants. In Cu-exposed plants, both miRNA398a and miRNA398b levels were reduced in the leaves of wild type plants. In the leaves of Cu-exposed oxi1 mutants, no significant changes in the expression levels of these miRNAs were detected.

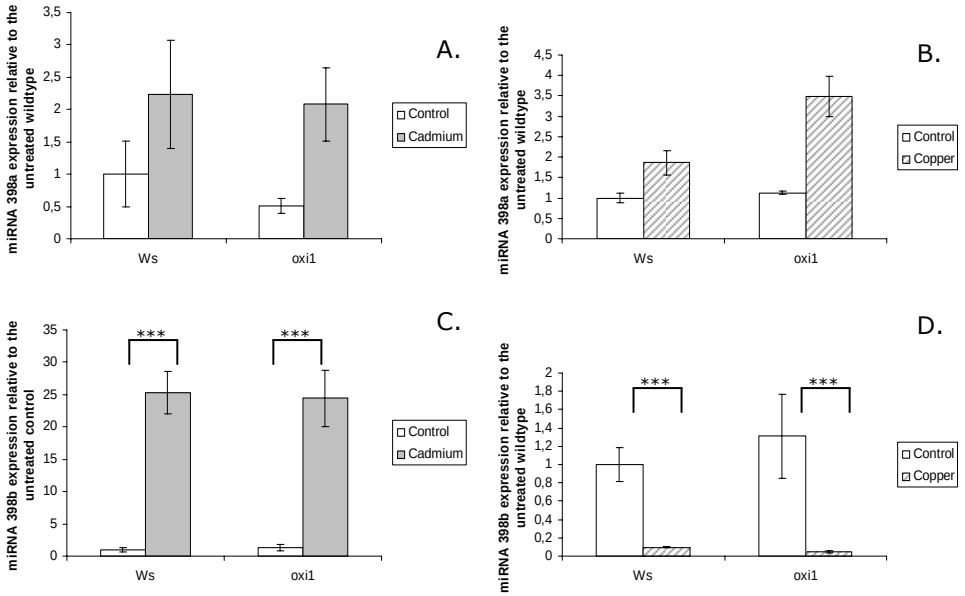


***Figure 10.5**: The expression level of miRNA398a (A+B) and miRNA398b (C+D) in the roots of 3-week-old Arabidopsis thaliana genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ over a 24h period or grown under control conditions (= 1). Values are mean ± S.E. of or 4 biological independent replicates (significance levels:*: p<0.1; **: p<0.05; ***: p<0.01).*

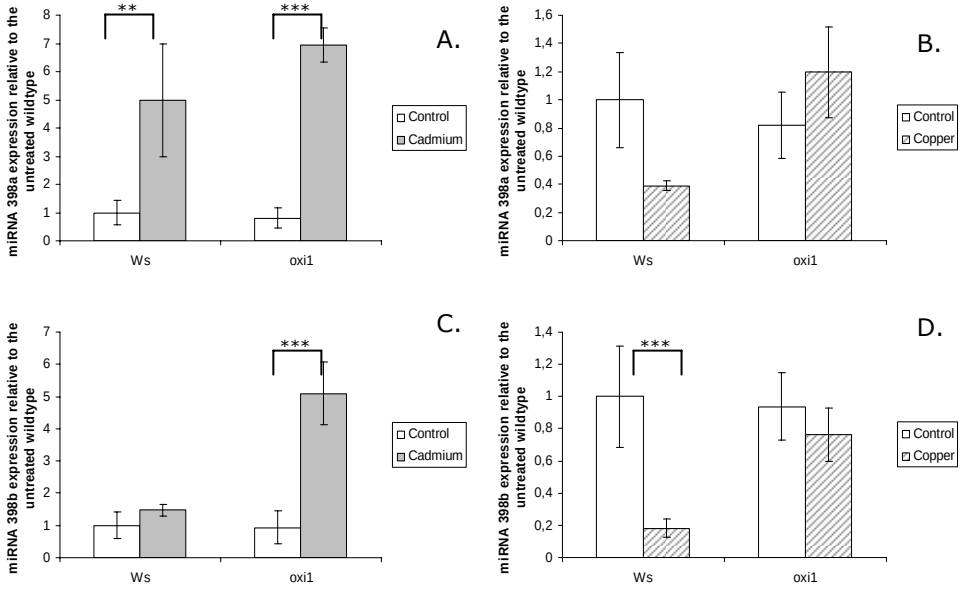


***Figure 10.6**: The expression level of miRNA398a (A+B) and miRNA398b (C+D) in the roots of 3-week-old Arabidopsis thaliana genotypes exposed to 5 $CdSO_4$ or 2 µM $CuSO_4$ over a 24h period or grown under control conditions (= 1). Values are mean ± S.E. of or 4 biological independent replicates (significance levels:*: p<0.1; **: p<0.05; ***: p<0.01).*

10.3 Discussion

The MAPK pathway is suggested as a central signalling cascade in multiple abiotic and biotic stress situations, among which heavy metal stress such as Cd and Cu (Jonak *et al.* 2004, Yeh *et al.* 2007). In the previous chapters, an important role for especially OXI1 in the Cd and Cu induced signalling was indicated by means of gene expression and clustering analyses. As this kinase is known as a key link between oxidative burst signals (such as H_2O_2) and downstream responses, it is highly important to unravel its specific role in heavy metal stress.

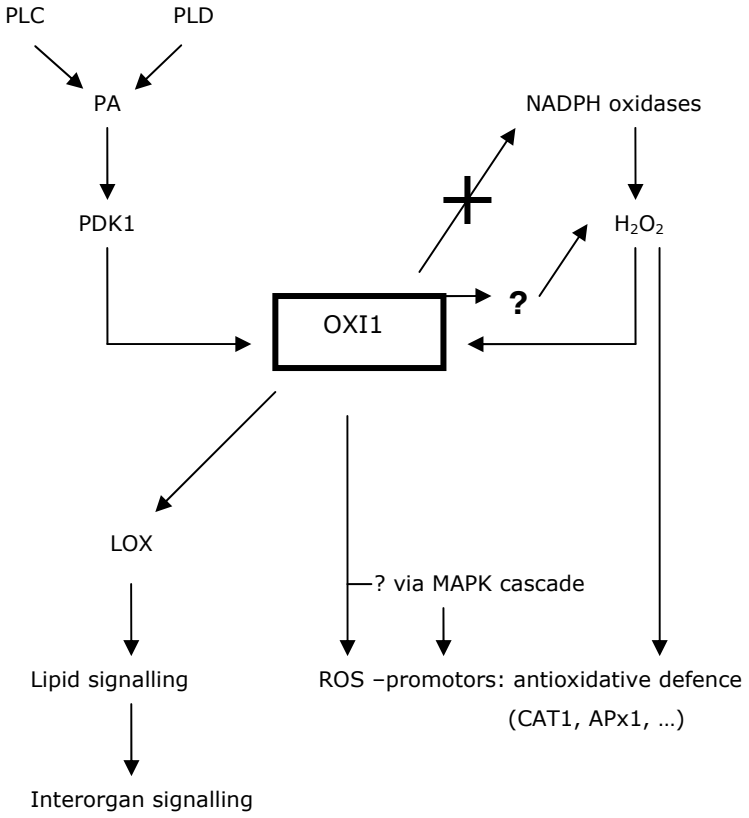


***Figure 10.7**: Proposed role of OXI1 (oxidative stress inducible kinase) in lipid and redox signalling networks. APX: ascorbate peroxidase; CAT: catalase; H_2O_2: hydrogen peroxide; LOX: lipoxygenase; PA: phosphatidic acid; PDK1: phosphoinositide dependent protein kinase; PLC: phospholipaseC; PLD: phospholipaseD*

Our results showed a root growth reduction in the *oxi1* mutants as a result of Cd and Cu exposure, although no growth reduction was observed in metal-exposed wild type plants (figure 10.1A). Therefore, OXI1 signalling seems important at the place of exposure, *i.e.* the roots. Other studies already suggested cell elongation, cell division and root differentiation as downstream OXI1 responses (Anthony *et al.* 2004) and these processes are probably strongly influenced due to the interaction of treatment (Cd/Cu) and *OXI1* knock-out in this study. H_2O_2, widely generated in many biological systems, is known to mediate root formation and development (Sasaki *et al.* 2005, Gajewska *et al.* 2006, Dunand *et al.* 2007, Li *et al.* 2007). The Cd- and Cu-related H_2O_2 reduction observed in the roots of the *oxi1* mutants, as compared to the metal-exposed wild type plants, together with the observed growth reduction (figure 10.1A and 10.2A), correlates with this hypothesis. Li *et al.* (2007). also demonstrated H_2O_2 as a promoter of root formation and growth in cucumber plants.

Former studies identified H_2O_2, and also PA (phosphatidic acid), as upstream components of OXI1 in the OXI1 signalling network (figure 10.7) (Anthony *et al.* 2006). Downstream signals of OXI1 are MPK3/6 together with the existence of a positive amplification loop, *i.e.* maintenance of H_2O_2 production via OXI1 activation (Mittler *et al.* 2004, Rentel *et al.* 2004). Disruption of this positive amplification loop and hence H_2O_2 production was confirmed by our results where a decrease in H_2O_2 levels was observed in the roots of metal-exposed *oxi1* mutants (figures 10.3 and 7). Whether NADPH oxidases exert a role in the H_2O_2-production downstream of OXI1 cannot be deduced from our results, as no clear changes in their expression patterns were observed between wild type plants and *oxi1* mutants (table 10.1). A clear investigation of all the NADPH oxidase isoforms under the conditions tested can add valuable information to our current understanding.

Besides H_2O_2, our results also identify LOXes as potential downstream targets of OXI1 (figure 10.7). *LOX1* upregulation was strongly reduced in the roots of both Cd and Cu-exposed *oxi1* mutants as compared to the roots of the metal-exposed wild type plants (table 10.1). A similar effect was observed in leaf *LOX2* expression that is normally induced by Cd (table 10.2). A key role for OXI1 in the complex network of lipid signalling was also demonstrated by Anthony *et al.* (2006). Moreover, PLD (phospholipase D) appears to be involved in the wound-

induced accumulation of jasmonic acid, with LOX2 as a downstream target through which PLD promotes the jasmonic acid production (Wang *et al.* 2000).

Overall, a role for OXI1 can be clearly hypothesized in the roots of Cu-exposed plants, as gene expression was strongly affected in the *oxi1* mutants under these conditions (table 10.1). Studies have shown *oxi1* mutants are impaired in the activation of the MAPKs MPK3 and MPK6 upon oxidative stress (Rentel *et al.* 2004). Complementary to these findings, decreased transcript levels of these MAPKs, but also of other regulatory proteins and antioxidative enzymes were observed in the roots of the Cu-exposed mutants. These results suggest a major role for OXI1 in the Cu-induced oxidative (defence) signalling that was not the case for Cd-exposed plants. Nevertheless, our data verify the importance of Ca^{2+}-signalling pathways during Cd stress in the roots of both wild type plants and *oxi1* mutants. Due to their chemical resemblance, a potential role for Ca^{2+} and its related components in the regulation of Cd-induced effects was also suggested in other studies (Garnier *et al.* 2006).

In the leaves of Cu-exposed plants, differences between genotypes were visible. Here, Cu caused an opposite effect as compared to the roots. Increases in the gene expression level of several antioxidative and regulatory genes were observed in the leaves of Cu-exposed *oxi* mutants (table 10.2). Presumably, the Cu-induced effects in the roots of these mutants influence interorgan regulatory mechanisms and induce root-to-shoot signalling (chapter 9). This hypothesis is supported by the metal concentration measurements, as Cu concentrations were not increased in the leaves of *oxi1* mutants (figure 10.2).

Besides MAPKs, Ca^{2+}-dependent kinases and transcription factors, also other regulatory mechanisms were differentially altered in the leaves of these Cu-exposed *oxi1* mutants. In normal circumstances, Cu induces a strong downregulation of miRNA398, which results in a strong upregulation of CuZnSOD expression (chapter 9) (Sunkar *et al.* 2006). In the leaves of *oxi1* mutants, however, the miRNA398 level did not change after Cu treatment and its relationship with CuZnSOD expression seemed disturbed (figure 10.6 B and D). It is possible that miRNA398 also regulates the expression of other genes, or CuZnSODs are regulated by different signalling networks, or both. As miRNAs seem more and more important in the complex network of cellular signalling, it

is of great importance to further unravel additional upstream and downstream targets of specific oxidative stress related miRNAs such as miRNA398.

In conclusion, our results indicate a highly important role for OXI1 in the cellular redox signalling during Cu-induced toxicity in the roots of *Arabidopsis thaliana*. Cadmium-exposed *oxi1* mutants were able to induce other signalling cascades, such as Ca^{2+}-dependent proteins, but no major role for OXI1 is hypothesized. H_2O_2 and LOXes were revealed as potential downstream targets of OXI1 in both stress situations, although other downstream components remain to be revealed in order to expand our knowledge regarding the exact role of OXI1 as a link between lipid and oxidative signalling during metal stress.

Chapter 11

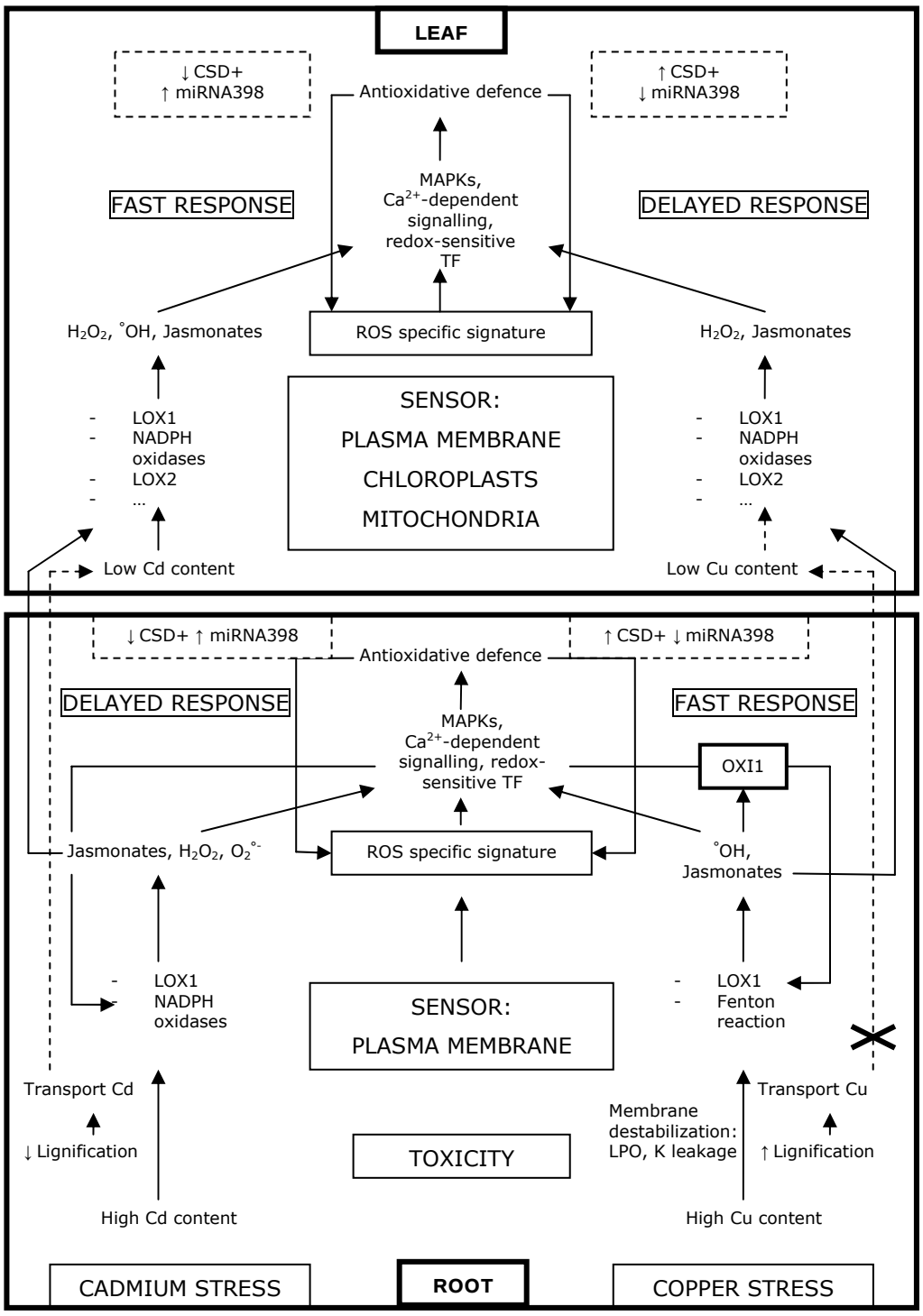
General discussion, conclusion and perspectives

11.1 Introduction

Elevated metal concentrations in the environment cause significant losses in crop production worldwide. Although the overall response to heavy metal exposure is quite similar and results in growth reduction and disruption of physiological processes such as photosynthesis and respiration, it is of great importance to expand our knowledge regarding the cellular and molecular processes influenced by metal toxicity. Moreover, when exploring the fundamental principles of cellular metal stress, characteristics and sequence of cellular events can lead to the discovery of metal-specific responses, and may be used as potential biomarkers.

The main objective of the present work concerns the cellular basis of heavy metal-induced responses, and more specific the role of oxidative stress herein. Oxidative stress is a disturbance of the cellular redox homeostasis and is a common phenomenon in multiple stress situations. It is a frequently studied process, but the knowledge regarding its underlying mechanisms within heavy metal toxicity is rather scarce. In this study, ROS (reactive oxygen species)-related processes were investigated in *Arabidopsis thaliana* after a 24h exposure to low metal concentrations. Metals (Cu, Cd) with distinct characteristics, e.g. essential versus non-essential, redox-active versus not redox-active, were supplied to the roots. Cellular responses were studied in both roots and shoots in conditions where different metal concentrations were compared, in a monometallic as well as in a multi-pollution context. In this way, both metal-specific as well as common stress effects were revealed. Figure 11.1 gives an overview of the involvement of oxidative stress as a modulator in the regulation of the cellular responses in *Arabidopsis thaliana* seedlings exposed to Cd or Cu.

Figure 11.1 *(p150): ROS specific signature is a dynamic process and acts as a modulator in the regulation of cellular responses under Cd and Cu stress.*


LEAF
↓ CSD+
↑ miRNA398
Antioxidative defence
↑ CSD+
↓ miRNA398
FAST RESPONSE
MAPKs,
Ca2+-dependent
signalling,
redox-sensitive
TF
DELAYED RESPONSE
H2O2, ˚OH, Jasmonates
ROS specific signature
H2O2, Jasmonates
- LOX1
- NADPH oxidases
- LOX2
- …
SENSOR:
PLASMA MEMBRANE
CHLOROPLASTS
MITOCHONDRIA
- LOX1
- NADPH oxidases
- LOX2
- …
Low Cd content
Low Cu content
↓ CSD+ ↑ miRNA398
Antioxidative defence
↑ CSD+ ↓ miRNA398
DELAYED RESPONSE
MAPKs,
Ca2+-dependent
signalling, redox-
sensitive TF
FAST RESPONSE
OXI1
Jasmonates, H2O2, O2˚-
ROS specific signature
˚OH,
Jasmonates
- LOX1
- NADPH oxidases
SENSOR:
PLASMA MEMBRANE
- LOX1
- Fenton reaction
Transport Cd
↓ Lignification
TOXICITY
Membrane
destabilization:
LPO, K leakage
Transport Cu
↑ Lignification
High Cd content
High Cu content
CADMIUM STRESS
ROOT
COPPER STRESS

11.2 Oxidative stress as a modulator during Cd and Cu toxicity in the roots of *Arabidopsis thaliana*

Copper is very toxic for the roots and exposure of 3-week-old *Arabidopsis thaliana* seedlings resulted in phytotoxic effects such as membrane destabilization in accordance with K-leakage, as well as growth reduction (chapter 5-6). The same effects, albeit to a lesser extent, were only observed after exposure to the highest Cd concentration (20 µM) (chapter 4). One of the underlying mechanisms probably is the induction of lipid peroxidation, strongly stimulated by Cu via the Fenton reaction (figure 9.3).

The plasma membrane often functions as a sensor in the first contact between metals and root cells (figure 11.1). Here, protein complexes play a crucial role in perceiving changes and induce downstream signalling responses. In case of Cd toxicity, this was demonstrated by the elevated NADPH-oxidase-dependent superoxide ($O_2^{\circ-}$) burst (chapter 7). In the Cd-exposed roots, $O_2^{\circ-}$ and H_2O_2 are suggested as important signalling molecules (figures 7.3 and 7.4), produced at the plasma membrane (NADPH oxidases). Copper, on the other hand, did not influence NADPH-oxidase expression, nor was a Cu-induced H_2O_2 response detected. When Cu enters the root cells, ROS-production can be directly catalyzed via Fenton and Haber-Weiss reactions, which probably explains the strong phytotoxic effect of this metal (Schutzendubel and Polle 2002). It is plausible that produced H_2O_2 in Cu-exposed plants is immediately converted to other ROS, such as the hydroxyl radical ($^{\circ}OH$).

Inside the cell, ROS are balanced against antioxidative defence mechanisms and ROS signalling is controlled by production and scavenging (Ott *et al.* 2002). ROS are able to activate downstream signalling cascades, and hence trigger cellular defence components. In both, Cd and Cu exposed roots, GSH-associated antioxidative defence was strongly activated at all exposure concentrations. Besides their antioxidative role, GSH-related enzymes also participate in controlling the cellular metal homeostasis (via GSH and/or phytochelatin production). Our results indicated a key role for GST2 and the transporter MRP4 in both Cd and Cu detoxification and sequestration (tables 7.2 and 7.3, figure 9.5). GSH-complexes can be transported to the vacuole via MRP4, although, as

a plasma-localized transporter, MRP4 may also function as an efflux mechanism (Klein *et al.* 2006). Also other antioxidative components such as CAT1 and APX2 were of similar significance in both stress situations. Metal-specific antioxidative responses were related to the SOD-expression, where FSD1 expression was strongly upregulated in the roots of the Cd-exposed plants, accompanied by a miRNA398-regulated CSD downregulation (figures 5.4 and 9.8, table 6.2).

Exposure to Cu resulted in an immediate activation of the signalling pathway at the transcriptional level. A more delayed, only at the highest exposure concentration, and less intense MAPK induction was observed in roots of Cd-exposed plants (table 7.2 and 9.1, figure 11.1); this confirms results obtained at the protein level in roots of *Medicago* plants (Jonak *et al.* 2004). A similar trend was observed for Ca^{2+}-dependent signalling components such as SOS3 and CDPK1 (table 7.2 and 9.1). ROS signatures possibly cause differences in co-expression patterns. Antioxidative genes were co-regulated during Cd stress (figure 7.5), whereas Cu strongly induced both antioxidative genes *(GST2, CAT1, …)* as well as regulatory genes *(OXI1, MPK3, CDPK1 …)* at all exposure concentrations (figure 9.4 and 5, table 9.1). We therefore hypothesize that under Cd stress, a fast response mechanism induces the transcription of several defence components, either via the phosphorylation of signalling cascades or directly by ROS themselves. In a later phase, MAPK- and Ca^{2+}-dependent components are upregulated to sustain the signalling network. Copper, on the other hand, is directly toxic to the roots as it induces $^{\circ}OH$ production via the Fenton reaction. Because of this uncontrolled oxidative burst, MAPK signalling components as well as defence mechanisms are instantly activated under Cu stress. A central role for OXI1 in the upregulation of both was demonstrated during Cu-exposure (chapter 10).

Whereas root-to-shoot transfer of Cd was restricted, a nearly complete retention of Cu in the roots, probably as a consequence of induced lignification (figure 9.2), was observed (figures 4.1, 9.2 and 11.1). Therefore interorgan communication should be involved, *i.e.* stress signals can be passed on, even before the metal arrives in the leaves. We hypothesize a significant role of LOX-dependent oxylipin and jasmonate as possible root-to-shoot signalling

molecules. ROS, produced by NADPH oxidases, might also be important in intercellular signalling under Cd stress, as an accurate long-distance transmission of ROS from roots to shoot was demonstrated in previous research (Capone *et al.* 2004, Yang *et al.* 2006).

11.3 Oxidative stress as a modulator during Cd and Cu toxicity in the leaves of *Arabidopsis thaliana*

In the leaves of metal-exposed plants, no visible stress effects nor morphological changes were observed and deterioration such as lipid peroxidation was only detected after an exposure to the highest Cu concentration (10 µM) (figure 6.1). Nevertheless, multiple molecular and biochemical processes were induced. Signalling molecules such as H_2O_2 and jasmonates, or the metal itself, especially in the case of Cd toxicity, can trigger sensors at the plasma membrane, chloroplasts and mitochondria (figure 11.1). Both metals strongly induce the expression of the plasma membrane-localized enzymes NADPH oxidase and LOX1 (figure 5.3, table 6.1), and of the chloroplast-localized enzyme LOX2. These enzymes produce intracellular signals (oxylipins, ROS) that activate specific defence and/or regulation mechanisms (figures 8.1 and 8.2, table 8.1). Fine-tuning of ROS levels is executed by its production and scavenging. Metal-specific effects, especially on SOD expression and activation profiles, may result in a metal-specific ROS signature, based on its constituents and places of ROS production (figures 5.4 and 9.8).

Secondary messengers induce the transcription of signal transduction and defence mechanisms, which was the case in both stress conditions. A strong simultaneous upregulation of ROS-producing, ROS scavenging and regulatory genes (figure 8.3) implies a transcriptional co-regulation of these genes under Cd stress. Cadmium-induced ROS (H_2O_2 and $^{\circ}OH$) cause a transcriptional induction of genes such as *OXI1, WRKY transcription factors, APX1* and *CAT1* directly via redox-sensitive DNA binding domains (Guan *et al.* 2000, Tosti *et al.* 2006). Besides a direct transcriptional induction, ROS can also activate signal transduction cascades at the protein level and a central signalling function for OXI1 was proposed in the leaves during Cd stress. Experiments with *oxi1* mutants, however, showed no altered expression patterns of above-mentioned genes. Cross talk between signalization pathways is common within various

types of (a)biotic stress situations (Kaur and Gupta 2005). Besides OXI1, other signalling components, such as Ca^{2+}-dependent kinases, clearly are important in the Cd-induced cellular responses. Due to their chemical resemblance, a potential role for Ca^{2+} and its related components in the regulation of Cd-induced effects was also suggested in other studies (Garnier *et al.* 2006).
Copper, on the other hand, only induces leaf antioxidative defence and signal transduction mechanisms in a later stage, *i.e.* at the highest exposure concentrations (figures 5.4 and 5.5, table 9.1). As Cu concentrations in the leaves remain rather similar between the different exposure concentrations, it is likely that when a certain stress level in the roots is reached, underlying interorgan signalling mechanisms induce specific responses in the leaves. This is also demonstrated in the leaves of the Cu-exposed *oxi1* mutants, as Cu-induced effects in the roots of these mutants influence interorgan regulatory mechanisms and induce gene expression of several antioxidative and regulatory genes in the leaves.

11.4 Conclusion and future perspectives

Organisms are usually exposed in a multipollution context. In this study, a better understanding of metal-specific induced stress responses was aimed.

Exposure of *Arabidopsis thaliana* seedlings to either metal clearly results in some common cellular responses, such as the induction of LOXes. In general, both metals also induce MAPK signalling cascades, but with emphasis on different components showing distinct expression patterns.
In the roots, metal-specific differences were related to the chemical properties of Cd or Cu. An NADPH-oxidase dependent ROS burst was observed under Cd stress that was absent under Cu stress. In both roots and leaves, interesting metal-specific markers were detected in the gene expression profile of superoxide quenching enzymes, and their related miRNA398 profiles.
Whether the cellular responses remain common or specific stress effects in a multipollution context are important topics for further investigation and should be extended to other pollutants. In addition, similarities and differences in regulation profiles can be used to detect metal-specific responses.

Cellular responses to heavy metal stress are an interplay of multiple pathways. The presented data demonstrate an organized cooperation between ROS-inducing, antioxidative and regulatory mechanisms as a result of the metal applied and for the organ studied. It is evident that signalling mechanisms in plants do not operate alone but that extensive cross talk occurs between signal transduction pathways in both stress situations. Similarly regulated transcription profiles of CDPKs, MAPKs and NADPH oxidases were demonstrated by means of clustering analyses, although the specific regulator(s) remain to be identified. Performing of kinetic experiments will deliver time-related induction patterns of the cooperating components; this should allow to unravel to the sequence of events at specific time points and/or cellular locations during metal stress.
Integration of direct metal-induced regulation and ROS-mediated regulation with components of intercellular signal transduction pathways was demonstrated. An important role for jasmonates and oxylipines as central intercellular signalling molecules was suggested, but also H_2O_2 might act as a long distance signalling component during heavy metal stress. It is of great interest to define the exact role of jasmonate, H_2O_2 and other components in the root-to-shoot signalling to complete our knowledge about long distance signalling during heavy metal stress.

In conclusion, the data obtained demonstrate that even under environmentally realistic metal concentrations, the signal sensing and hence the cellular response is strongly dependent on the stress intensity and the chemical properties of the metal applied. Clear differences in cellular responses were also observed between roots and shoots, especially when studying signal transduction cascades. In order to explain specific metal-related responses, or to screen for potential biomarkers, study of underlying molecular mechanisms, especially processes related to signal transduction are important topics for future research. In this study, we mainly focused on the transcriptional changes in the cellular response, but it is highly recommended to extend this knowledge by studying signalization processes at genome (methylation studies), proteome and metabolomic level.

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