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DOCTORAL DISSERTATION

Oxidative signaling in *Arabidopsis thaliana* plants: The role of ascorbate and ascorbate peroxidases during cadmium or copper toxicity

Doctoral dissertation submitted to obtain the degree of doctor of Science: Biology, to be defended by

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WOORD VOORAF

Het schrijven van een doctoraat is als een riskante lange reis vol avonturen, waarin ik heel wat nieuwigheden heb ontdekt, topmomenten heb beleefd, maar ook door dalletjes ben gegaan. Het einde van deze mooie en avontuurlijke reis staat voor de deur en ik kijk terug op een zeer leuke tijd. Uiteraard zou ik hier nu niet staan zonder de hulp en de steun van enorm veel mensen!

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SUMMARY

Over the past decades, large areas have been contaminated with cadmium (Cd) and copper (Cu) due to anthropogenic activities. These toxic metals are able to accumulate in plants where they interfere with essential physiological processes necessary for normal functioning. A common cellular consequence of toxic metal exposure is an increase of reactive oxygen species (ROS), thereby disturbing the cellular redox status and causing oxidative damage to cellular components. However, controlled levels of ROS can also act as signaling molecules in normal cell metabolism as well as in defense responses. Hydrogen peroxide (H_2O_2) is a well-known example involved in the control of such responses and its production is elevated in plants exposed to Cd or Cu. Therefore, H_2O_2 could be involved in metal-induced signal transduction events. Ascorbate peroxidases (APX) are important H_2O_2 scavengers and show a high affinity in the presence of ascorbate (AsA). Therefore APXs are involved in protecting the plant cell against harmful amounts of H_2O_2 as well as controlling H_2O_2 levels for signaling involved in defense responses.

To understand plant responses to toxic concentrations of metals, it is necessary to gain knowledge about stress signaling caused by this stress factor at the cellular level. Because AsA and APX are involved in controlling H_2O_2 levels, they play a role in stress signaling pathways hence in many cellular processes. However, until today, a potential role for APX and AsA in biological processes dealing with metal-induced oxidative stress and downstream responses in plants has not been studied. Therefore, the main objective of this work was to unravel the role of APX and AsA in oxidative signaling and in the regulation of oxidative stress responses during Cu and Cd exposure.

In the first part of this work, the function of 2 cytosolic isoforms of APX, APX1 and APX2, were investigated in Cu- and Cd-induced oxidative stress using knockout *apx1* (*ko-apx1*) and knockout *apx2* (*ko-apx2*) *Arabidopsis thaliana* plants (Chapter 4 and 5). After prolonged (72 h) Cu exposure, both *ko-apx* mutants seemed to acclimate and cope better with Cu stress, compared to

wildtypes, as they show higher fresh weights in roots and leaves, possibly due to an altered signaling pathway. Indeed, *ko-apx* roots showed a suppressed signaling and a diminished expression of ethylene biosynthesis genes indicating that APX1 and APX2 are needed for a proper signal transduction leading to ethylene synthesis. A reduction in ethylene synthesis may be linked to a small growth recovery in both mutants upon Cu exposure that was not present in wild-type plants. Also in the leaves, changes in the downstream Cu-induced responses were observed. An increased synthesis of AsA was noticed in *ko-apx2* mutants; on the other hand, Cu-exposed *ko-apx1* leaves showed a constitutively higher expression of transcription factor *ZAT7*. This eventually could lead to the activation of plant defenses, and as such to higher biomass production as compared to wild-type plants. In the case of Cd, mainly APX1 is involved and required for normal signal transduction and defense responses. Consequently, a suppressed expression of oxidative signaling genes was observed in Cd-exposed *ko-apx1* roots, which in turn gives rise to a diminished response of the antioxidative defense system. The suppressed root signal transduction in *ko-apx1* plants could result in alterations in root-shoot signaling leading to lower H_2O_2 levels and hence affecting leaf defense responses. Consequently, an altered ethylene biosynthesis, lower GSH levels and lower antioxidant enzyme activities were indeed noticed in *ko-apx1* plants exposed to Cd.

In the following part (Chapter 6), the role of AsA and GDP-D-mannose pyrophosphorylase (GMP; involved in AsA synthesis and cell wall processes) in Cd- and Cu-induced stress responses and signal transduction pathways were examined in mutants with reduced AsA levels (*vtc1-1* and *vtc2*). In case of Cu exposure, no prominent differences between *vtc1-1* and wild-type plants could be identified. On the other hand, Cd exposure resulted in reduced growth and membrane damage already after 24 h in *vtc1-1*, indicating that the involvement of GMP in cell wall processes as well as AsA itself were important in provoking responses at this level. Furthermore, a reduction in the expression of signaling genes was observed in roots and leaves of *vtc1-1*, probably due to decreased levels of H_2O_2. In turn, this could lead to a lack of induction of pro-oxidative genes and a lack of changes in antioxidative gene expression. A low AsA level as well as a deficient GMP activity are thus both responsible for a higher sensitivity

of plants to Cd (decreased leaf fresh weight) and are both involved in Cd-induced signaling pathways.

Finally, the role of APX1, APX2 and AsA was examined after long-term metal exposure (Chapter 7). The observed higher tolerance of *ko-apx* mutants after short-term Cu exposure was of short duration as they show a similar inhibition of rosette growth and length of inflorescence as wild-type plants after long-term exposure, although a delayed onset of the inflorescence emergence was noticed. In the case of Cd, a lack of APX2 resulted in a better vegetative growth, while APX2 was needed for the reproductive tissues.

In conclusion, the results obtained in this work are indicative of an essential role for APX1/2 and AsA in Cd- and Cu-induced signaling pathways resulting in oxidative stress responses. Knowledge about the metal-induced responses is essential to understand how plants acclimate and cope with elevated metal concentrations at the physiological level.

SAMENVATTING

In de afgelopen decennia werden grote gebieden verontreinigd met cadmium (Cd) en koper (Cu) als gevolg van humane activiteiten. Deze toxische metalen kunnen accumuleren in planten en interfereren met essentiële fysiologische processen die noodzakelijk zijn voor een normale plantontwikkeling. Een algemeen cellulair gevolg van blootstelling aan toxische metalen is een verhoging van reactieve zuurstofvormen (ROS), waardoor de cellulaire redox toestand wordt verstoord en oxidatieve schade aan cellulaire componenten optreedt. Daarnaast hebben gecontroleerde niveaus van ROS ook een belangrijke rol als signaalmolecule zowel in het controleren van het normale celmetabolisme alsook verdedigingsresponsen. Waterstofperoxide (H_2O_2) is een bekend voorbeeld betrokken bij de controle van deze responsen en de productie ervan wordt aanzienlijk verhoogd in planten blootgesteld aan Cd en Cu. Hierdoor is het mogelijk dat H_2O_2 betrokken is in metaal-geïnduceerde signalisatie netwerken. Ascorbaatperoxidasen (APX) zijn belangrijke scavengers van H_2O_2 en vertonen een hoge affiniteit voor H_2O_2 in aanwezigheid van ascorbaat (ASA). Hierdoor staan APXs in voor een bescherming van de plantencel tegen schadelijke hoeveelheden H_2O_2 alsook voor de regulatie van de hoeveelheid H_2O_2 voor signalisatie betrokken in verdedigingsresponsen.

Om te kunnen begrijpen hoe planten reageren op toxische metaalconcentraties, is het noodzakelijk om meer inzicht te krijgen in de signaaltransductie pathways die deze stress-factor op cellulair niveau veroorzaakt. Omdat AsA en APX betrokken zijn in de regulatie van de hoeveelheid H_2O_2, spelen ze een rol in signaaltransductie pathways en dus in vele cellulaire processen. Een mogelijke rol voor APX en AsA in biologische processen die omgaan met metaal-geïnduceerde oxidatieve stress en downstream responsen in planten zijn echter tot op heden nog niet onderzocht. Daarom is de algemene doelstelling van deze doctoraatsstudie om de functie van APX en AsA te ontrafelen in oxidatieve signalisatie en in de regulatie van oxidatieve stress responsen tijdens Cu en Cd blootstelling.

Samenvatting

In het eerste deel van dit werk werd de functie van 2 cytosolaire APX isovormen, APX1 en APX2, in Cu en Cd geïnduceerde oxidatieve stress onderzocht met behulp van knockout *apx1* (*ko-apx1*) en knockout *apx2* (*ko-apx2*) *Arabidopsis thaliana* planten (Hoofdstuk 4 en 5). Na lange (72 h) Cu blootstelling lijken beide *ko-apx* mutanten beter om te gaan met Cu stress in vergelijking met wild-type planten vermits ze een hoger versgewicht vertonen in wortels en blaadjes, mogelijk als gevolg van een veranderde signaaltransductie pathway. De wortels van *ko-apx* mutanten vertoonden inderdaad een verminderde signalisatie en daarbij een verminderde expressie van ethyleenbiosynthese genen wat suggereert dat APX1 en APX2 nodig zijn voor een goede signaaltransductie die leidt tot ethyleensynthese. Een vermindering in de synthese van ethyleen kan worden gekoppeld aan een klein groeiherstel in beide mutanten die niet te zien was in wild-type planten. Ook in de blaadjes werden veranderingen waargenomen in de downstream Cu geïnduceerde responsen. Een verhoogde synthese van AsA werd opgemerkt in *ko-apx2* mutanten; anderzijds vertoonden Cu blootgestelde *ko-apx1* blaadjes een constitutief hogere expressie van de transcriptiefactor *ZAT7.* Dit kan uiteindelijk leiden tot de activatie van verdedigingsresponsen, die mee aan de basis liggen van een hogere biomassaproductie in vergelijking met wild-type planten. Tijdens Cd blootstelling is voornamelijk APX1 betrokken in en nodig voor normale signaaltransductie en verdedigingsresponsen. Bijgevolg werd een verminderde expressie van oxidatieve signalisatiegenen waargenomen in Cd blootgestelde *ko-apx1* wortels, wat op zijn beurt aanleiding geeft tot een verminderde respons van het antioxidatief verdedigingssysteem. De waargenomen verminderde signaaltransductie in *ko-apx1* wortels kan resulteren in veranderingen in wortel-blad signalisatie die leiden tot lagere H_2O_2 niveaus en daarmee invloed hebben op de verdedigingsresponsen van het blad. Een veranderde ethyleenbiosynthese, lagere GSH niveaus en lagere activiteiten van antioxidatieve enzymen werden inderdaad opgemerkt in *ko-apx1* planten blootgesteld aan Cd.

In het volgende deel (Hoofdstuk 6) werd de rol van AsA en GDP-D-mannose pyrofosforylase (GMP; betrokken bij AsA synthese en celwandprocessen) in Cd en Cu geïnduceerde stressresponsen en signaaltransducties onderzocht in

mutanten met verminderde AsA niveaus (*vtc1-1* en *vtc2*). Tijdens Cu blootstelling werden geen zichtbare verschillen tussen *vtc1-1* en wild-type planten geïdentificeerd. Anderzijds resulteerde Cd blootstelling al na 24 u in een verminderde groei en een gestegen membraanschade in *vtc1-1* planten, wat aangeeft dat de betrokkenheid van GMP in celwandprocessen evenals AsA zelf belangrijk zijn in het uitlokken van responsen op dit niveau. Bovendien werd een vermindering in de expressie van de signalisatiegenen waargenomen in wortels en blaadjes van *vtc1-1* mutanten, vermoedelijk veroorzaakt door verlaagde niveaus van H_2O_2. Dit op zijn beurt kan leiden tot een verminderde inductie van pro-oxidatieve genen en een gebrek aan veranderingen in de expressie van antioxidatieve genen. Een laag AsA niveau evenals een deficiënte GMP activiteit zijn dus beiden verantwoordelijk voor een hogere gevoeligheid van planten voor Cd (vermindering van het blad versgewicht) en zijn beide betrokken bij Cd geïnduceerde signaaltransductie pathways.

Ten slotte werd de rol van APX1, APX2 en AsA onderzocht na langdurige (verschillende weken) metaalblootstelling (Hoofdstuk 7). De waargenomen hogere tolerantie van *ko-apx* mutanten na 72 h Cu blootstelling was van korte duur vermits ze vergelijkbare inhibities van de rozetgroei en lengte van de bloeiwijze vertoonden als wild-type planten na langdurige blootstelling. Hoewel een vertraagd uitlopen van het meristeem van de bloeiwijze werd opgemerkt in de mutanten. Afwezigheid van APX2 tijdens Cd blootstelling resulteerde in een betere vegetatieve groei, terwijl APX2 nodig was voor de reproductieve weefsels.

Alle resultaten van dit werk tonen aan dat APX1/2 en AsA een essentiële rol spelen in Cd en Cu geïnduceerde signalisatie pathways die resulteren in oxidatieve stress responsen. Kennis over de metaal geïnduceerde responsen is essentieel om te begrijpen hoe planten op fysiologisch niveau omgaan met verhoogde metaalconcentraties.

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LIST OF ABBREVATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
Al	aluminium
AO	ascorbate oxidase
AOX	alternative oxidase
APX	ascorbate peroxidase
As	arsenic
AsA	ascorbate
Ca	calcium
cAPX	cytosolic ascorbate peroxidase
CAT	catalase
Cd	cadmium
chlAPX	chloroplastic ascorbate peroxidase
Cr	chromium
CSD	copper/zinc superoxide dismutase
CTR	conserved copper transporter family
Cu	copper
DAB	3,3’-diaminobenzidine
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DW	dry weight
ERF1	ethylene response factor 1
Fe	iron
FSD	iron superoxide dismutase
FW	fresh weight
G6PDH	glucose-6-phosphate dehydrogenase
GalDH	L-galactose dehydrogenase
GalLDH	L-galactone-1,4-lactone dehydrogenase
GalPP	L-galactose-1-P-phosphatase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLGalPP	GDP-L-galactose phosphorylase
GME	GDP-mannose-3',5'-epimerase
GMP	GDP-mannose-pyrophosphorylase
GPX	guaiacol peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
H_2O	water
H_2O_2	hydrogen peroxide

Abbrevations

HSF	heat shock factor
ICDH	NADP-dependent isocitrate dehydrogenase
LOX	lipoxygenase
MAPK	mitogen-activated protein kinase
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
ME	malic enzyme
Mg	magnesium
miRNA398	microRNA398
mitAPX	mitochondrial APX
Mn	manganese
MSD	manganese superoxide dismutase
NADPH	nicotinamide adenine dinucleotide
Ni	nickel
$^1O_2$	singlet oxygen
O_2	atmospheric oxygen
$O_2°^-$	superoxide
°OH	hydroxyl radical
OX	oxidative stress marker
OXI1	oxidative-signal inducible 1
PAL	phenylalanine ammonia-lyase
Pb	lead
PCR	polymerase chain reaction
PGI	phosphoglucose isomerase
PMI	phosphomannose isomerase
PMM	phosphomannomutase
PSI/II	photosystem I/II
PUFA	polyunsaturated fatty acid
RBOHC/D	respiratory burst oxidase homologue C/D
ROS	reactive oxygen species
SAGs	senescence-associated genes
SDGs	senescence-down-regulated genes
SOD	superoxide dismutase
SPX	syringaldazine peroxidase
Sr	strontium
TBArm	thiobarbituric acid reactive metabolites
tAPX	thylakoid ascorbate peroxidase
VDE	violaxanthin de-epoxidase
Vtc	vitamin C
WRKY22/25	WRKY transcription factor
ZAT7/10/12	zinc finger transcription factor
Zn	zinc

CHAPTER 1
Introduction

An Bielen, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2013). The influence of metal stress on the availability and redox state of ascorbate, and possible interference with its cellular functions. International journal of molecular sciences 14, 6382–6413.

1.1. Cadmium and copper

Cadmium (Cd) and copper (Cu) are naturally occurring in the environment, at relatively low concentrations, as a consequence of weathering of sedimentary rocks or are emitted in the atmosphere by eruptions of volcanoes or by forest fires. However, the major causes that have led to high levels of metals in the environment are anthropogenic releases (Benavides *et al.* 2005, Yruela 2005). For example, Cu is emitted by mining, smelting of metals and by the use of agricultural technologies (Nagajyoti *et al.* 2010). Cadmium is released into the environment by urban traffic or as a by-product of zinc (Zn) mining and smelting, as Cd naturally occurs in association with Zn ores (Vig 2003, Nagajyoti *et al.* 2010). Consequently, the metal industry has led to increased diffusion of metals into the environment, which has become an important process in the geochemical cycling of these elements (Islam *et al.* 2007). At the end of the last century, metal industries switched to more environmentally friendly production processes, resulting in a decreased release of metals into the environment. However, in many regions, historical pollution still causes problems, while in developing countries, pollution still continues (Lauwerys *et al.* 1990, Sanità di Toppi and Gabrielli 1999, Nawrot *et al.* 2008). Metals can cause serious problems for all organisms when their natural occurrence is exceeded. They can accumulate in crops grown on metal-contaminated soils with negative consequences for the quality and safety of feed and food crops. This in turn is strongly favoring food chain contamination and thus human health (Islam *et al.* 2007, Bernard 2008, Cockell *et al.* 2008).

In humans, food, drinking water, and Cu-containing diet supplements are the main sources of Cu exposure, whereas intake of Cu through inhalation or dermal routes is minimal for most people (Cockell *et al.* 2008, de Romana *et al.* 2011).

Early adverse effects after acute exposure to Cu originate in the stomach, eliciting a reflex response of nausea and vomiting (de Romana *et al.* 2011). Chronic ingestion of high levels of Cu results in Cu accumulation in the liver, ultimately causing liver dysfunction (Cockell *et al.* 2008, de Romana *et al.* 2011). The main and best-known example of chronic Cu toxicity is Wilson's disease, an autosomic recessive genetic disorder, characterized by the accumulation of Cu. Most frequently, Wilson patients manifest chronic liver disease and neurological or psychiatric impairment frequently accompanied with kidney malfunction (de Romana *et al.* 2011). However, humans cannot live without Cu. Copper deficiency is more likely to occur early in life, especially in premature infants who have increased Cu requirements due to rapid growth, while their liver stores are reduced. Low Cu status has been associated with bone malformation during development, risk of developing osteoporosis in later life, poor immune responses and increased frequency of infections, poor cardiovascular health and alterations in cholesterol metabolism. Disturbance of the metabolism of other trace elements is also an effect of Cu deficiency, which may lead to secondary iron deficiency and anemia (de Romana *et al.* 2011).

Exposure to Cd occurs through ingestion of contaminated food or through inhalation of contaminated house dust or fumes and cigarette smoke (Lauwerys *et al.* 1990, Nawrot *et al.* 2006, Nawrot *et al.* 2008, Bernard 2008, Jarüp and Akkesson 2009). Tobacco plants can accumulate Cd from the soil, eventually resulting in a significantly higher level of Cd in the blood of smokers (Joseph 2009). Contaminated food is another important source of Cd exposure in the non-smoking population (Järup 2003). The exposure route greatly determines the extent to which Cd is absorbed; only 3 to 10 % of the ingested Cd is absorbed in contrast to 50 % of the inhaled Cd. Once Cd is taken up in the body, it is transported by the cardiovascular system to various organs (Jarüp and Akkesson 2009). The kidneys are by far the most important organs on which Cd exerts its toxicity as they efficiently retain Cd. As a consequence, Cd is nephrotoxic and can initially cause kidney tubular damage, eventually leading to renal failure. Other consequences of Cd nephropathy are bone demineralization, formation of kidney stones and bone fractures because of disturbances in calcium and phosphate metabolism (Bernard 2008, Joseph 2009, Nair *et al.* 2013). Cadmium exposure can also affect lung function and increases the risk of

lung cancer (Bernard 2008, Nawrot *et al.* 2006, Joseph 2009). Other organs that are affected by Cd are liver, testis, spleen, heart, thymus, salivary glands, epididymis and prostate (Joseph 2009). An important toxicological property of Cd is its exceptionally long half-life in the human body which can be as long as 20 years. Because of all the aforementioned information, the International Agency for Research on Cancer (IARC) classified Cd in 1993 as a human category I carcinogen (Jarüp 2003, Waisberg *et al.* 2003, Benavides *et al.* 2005, Jarüp and Akkesson 2009). Although the complete pathology evoked by Cd toxicity is unknown, the ability of Cd to elicit an oxidative stress response is apparent. Therefore animals and humans posses an antioxidant system which partially dependents on the absorbed food, because they lost the ability to synthesize themselves (Nair *et al.* 2013).

1.2. Plant responses to cadmium and copper exposure

As for humans, Cu is also an essential micronutrient for plants. It is required for normal plant growth and development as it participates in numerous physiological processes and is an essential cofactor for many metalloproteins. Excess Cu can disturb normal development by adversely affecting physiological processes in plants. A reduced biomass production, especially in the roots, has commonly been observed in plants exposed to Cu. Furthermore, Cu causes lipid peroxidation, decreases in lipid content and changes in fatty acid composition of membranes. When this happens in the thylakoid membranes, photosystem (PS) activity is seriously impaired (Yruela 2005).

In contrast, the non-essential nature of Cd makes this element toxic to plants, even at low concentrations, and negatively affects plant growth and development. Leaf necrosis, chlorosis and stunting are the main and most easily visible symptoms of Cd toxicity in plants. Furthermore, stomatal opening, transpiration and photosynthesis have been reported to be affected by Cd (Benavides *et al.* 2005, Cosio *et al.* 2006).

1.2.1. Bioavailability, uptake and transport of cadmium and copper in plants

In plants, metal uptake occurs mainly by the roots from soil and water. Rather little is taken up directly from the atmosphere via the leaves (Clemens 2006).

The uptake of metals depends on their bioavailability and concentration in the soil, as well as on the plant species (Santi di Topi and Gabrielli 1999, Nagajyoti *et al.* 2010). The bioavailability and concentration of Cd and Cu vary greatly with soil properties like pH, cation exchange capacity, temperature, concentration of organic compounds or other elements (Nagajyoti *et al.* 2010).

The acquisition of Cu by roots depends on specific high affinity Cu^+ uptake transporters, located at the plasma membrane and belonging to the conserved copper transporter (CTR) family. In *Arabidopsis thaliana*, the CTR family is known as copper transporter (COPT), which is involved in Cu acquisition from the exterior into the cytoplasm of plant cells (Puig *et al.* 2007). Since Cd is non-essential to the plant, no specific transporters were found for this metal. However, Cd resembles other essential divalent cations such as calcium (Ca), iron (Fe), manganese (Mn) and Zn, and highjack use the respective transporter systems for those elements (Verbruggen *et al.* 2009, Gallego *et al.* 2012). Furthermore, the uptake of cations is enhanced by the negative membrane potential inside the plasma membrane (Santi di Topi and Gabrielli 1999, Benavides *et al.* 2005). Once Cd and Cu are taken up by the roots, they can form complexes with chelators and chaperones, such as organic acids, phytochelatins or metallothioneins, which are involved in the intracellular trafficking of metal ions as well as metal detoxification (Benavides *et al.* 2005, Puig *et al.* 2007, Gallego *et al.* 2012). Excess Cu ions are mostly retained in the roots, whereas Cd taken up by the roots is transported to different plant organs (Benavides *et al.* 2005, Peralta-Videa *et al.* 2009).

1.2.2. Toxicity in plants

A good knowledge of plant–metal interactions is important for reducing the risks associated with the introduction of metals into the food chain (Benavides *et al.* 2005, Cuypers *et al.* 2009, Gallego *et al.* 2012, Cuypers *et al.* 2012a). At the morphological level, metal stress leads to visibly reduced plant growth due to reduced cell elongation and cell wall elasticity (Stoyanova *et al.* 2002, Benavides *et al.* 2005, Yruela 2005). A general scheme of cellular effects of toxic metals is shown in Figure 1.1. One of the main reasons of metal toxicity is their interaction with thiol-, histidyl- and carboxyl-groups of proteins, inhibiting structural, catalytic and transport functionality of the cell. The displacement of

essential cations from specific binding sites by toxic metals is another reason why metals can lead to inhibition or denaturation of proteins (Peralta-Videa *et al.* 2009, Sharma and Dietz 2009). Lastly, an important cellular response to an increased level of metals in plant cells is the disturbance of the cellular redox balance leading to oxidative stress (Fargasova 2001, Smeets *et al.* 2009, Cuypers *et al.* 2011) (see section 1.3).

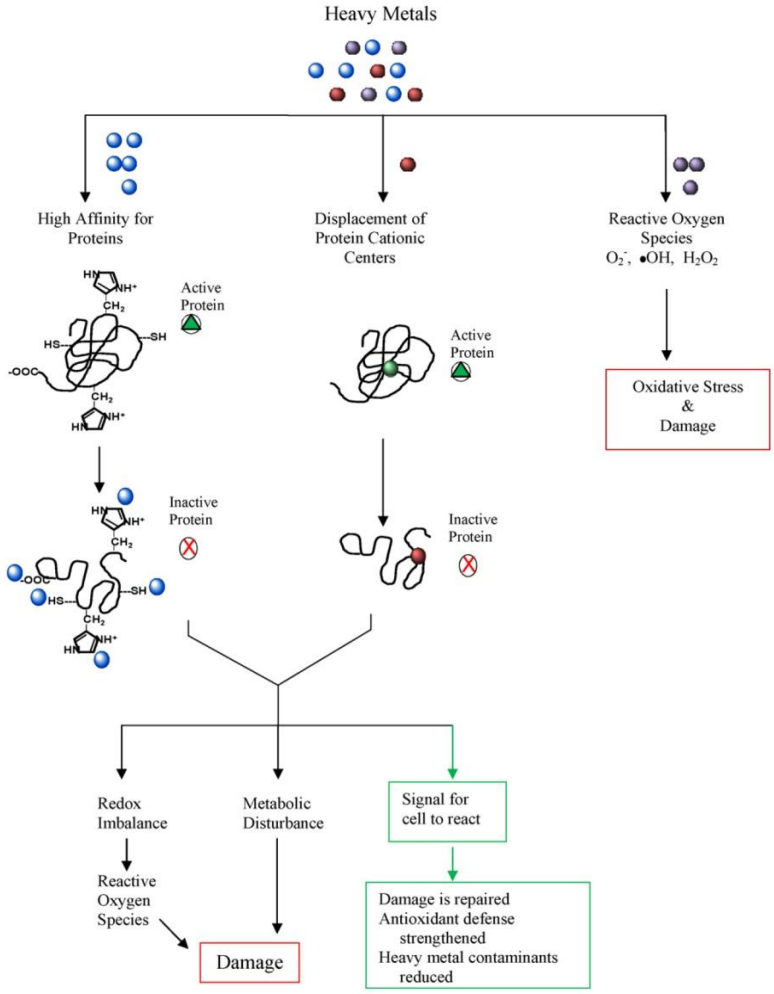

Figure 1.1. Heavy metal toxicity in plants (source: Peralta-Videa *et al.* 2009). Purple spheres indicate redox-active metals and red and blue are redox inactive metals. The green sphere is a metal center that is displaced by a heavy metal (red). The affinity for toxic metals will alter the activity of the protein and create imbalances and disruption that will lead to macromolecular damage. However the cell may adjust to the toxic metals and signal for reaction to prevent damage.

1.3. Oxidative stress

Exposure of plants to (a)biotic stresses can induce oxidative stress, a process in which the concentration of reactive oxygen species (ROS) is increased, leading to a disturbance of the equilibrium between ROS production and scavenging in favor of the former. Because of the differences in chemical characteristics between metals, they can cause oxidative stress in a direct or indirect way. Redox-active metals such as Cu can directly induce ROS production via Fenton and Haber–Weiss reactions (Figure 1.2) (Yruela 2005). In contrast, redox-inactive metals such as Cd induce ROS production only through indirect mechanisms such as inhibition of antioxidative enzymes or stimulation of ROS-producing enzymes such as NADPH oxidases (Stoyanova *et al.* 2002, Smeets *et al.* 2008b). When plants are exposed to metals, the increased levels of ROS can react with biological molecules leading to a damaged structure and inhibited function of the molecule. Reactive oxygen species are also produced during normal cell metabolism, and have a role in the regulation of growth, development and defense pathways (Mittler *et al.* 2004). Thus, excess levels of ROS can harm cellular structures and molecules, while controlled levels of ROS can act as signaling molecules to adjust development and induce defense responses. Plant cells contain an antioxidative system to scavenge ROS and maintain them within physiologically functional levels and to defend themselves against excessive levels of stress-induced ROS.

1.3.1. ROS production

During the production of ROS, molecular oxygen (O_2) can be converted to singlet oxygen ($^1O_2$), superoxide ($O_2°^-$), hydrogen peroxide (H_2O_2) or hydroxyl radical (°OH) (Halliwell 2006). Singlet oxygen is generated by an input of energy that rearranges the electrons, causing spin restriction. Therefore, the oxidizing ability is greatly increased and $^1O_2$ can directly oxidize proteins, DNA and lipids. Furthermore, ROS can be produced by the reduction of O_2, which is used as a terminal electron acceptor by aerobic organisms. In a first step, it is reduced to $O_2°^-$, which is moderately reactive. Further reduction of $O_2°^-$ leads to the formation of H_2O_2, which is not a free radical but a relatively stable molecule with a longer half-life than $O_2°^-$. It can migrate from the site of synthesis to other compartments and neighbouring cells by easily passing the membranes

(Halliwell 2006). However, $O_2°^-$ and H_2O_2 can change into the highly reactive °OH via the Fenton and Haber Weiss reaction. As shown in Figure 1.2, a redox-active metal like Cu can participate in this reaction to produce ROS directly. Hydroxyl radicals are the strongest oxidizing agents known and can initiate lipid peroxidation and attack DNA, proteins and many other molecules (Arora *et al.* 2002). The oxidation of organic substances may proceed via two possible reactions: the addition of °OH to the organic molecule or the substraction of a H^+ from it (Arora *et al.* 2002).

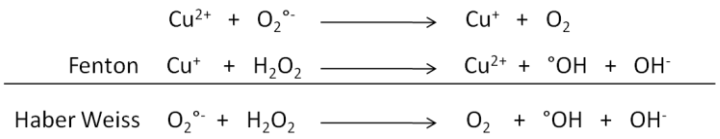

Figure 1.2. Fenton and Haber-Weiss reaction. The reactions are represented using Cu as an example.

Whereas under normal growth conditions the production of ROS in cells is low, metal stresses that disrupt the cellular homeostasis enhance the production of ROS (Mittler *et al.* 2002). Organelles with a strong oxidizing, metabolic activity, or with an intense speed of electron flow, such as chloroplasts and mitochondria, are major sources of ROS production in plant cells. Both chloroplasts and mitochondria are associated with redox sensing and signaling and are integrated in networks that are highly important in the regulation of cellular processes in both normal and stress conditions (Foyer and Noctor 2003). Another source of ROS production are NADPH oxidases located at the plasma membrane that catalyzes the reduction of O_2 to $O_2°^-$ via a transmembrane electron transport. NADPH oxidases play an important role in cellular responses against biotic and abiotic stresses, and they can function as intercellular signalling molecules to create ROS transients, possibly via the generation of secondary messenger H_2O_2 (Maksymiec 2007, Sharma and Dietz 2009). Additionally lipoxygenases (LOX) can be involved in ROS production. They catalyze the dioxygenation of polyunsaturated fatty acids (PUFAs) producing hydroperoxy fatty acids. When the hydroperoxy derivatives of PUFAs degrade, they can produce radicals that in turn oxidize biological components. Furthermore, LOX can mediate the formation

of $^1O_2$ and $O_2°^-$ (Porta and Rocha-Sosa 2002). On the other hand, LOX activity also forms precursors for membrane lipid derived signaling molecules called oxylipins, of which jasmonates are an example (Montillet *et al.* 2004, Ali *et al*. 2005).

It has been demonstrated that in plants, toxic metals lead to the production of ROS (Cuypers *et al.* 2011, Opdenakker *et al.* 2012a, Smeets *et al.* 2013). This increase is possibly due to elevated activities of NADPH oxidases and LOXs, since the expression levels of *RBOHC/D* (respiratory burst oxidase homologue C/D) (2 µM Cu), *RBOHD* (5 µM Cd), *LOX1* and *LOX2* (2 µM Cu and 5 µM Cd) were significantly increased. Remans *et al.* (2010) also observed increased expression levels of NADPH oxidases and LOXs after Cd and Cu exposure. The increased levels of ROS due to NADPH oxidases and LOXs may cause cellular damage, while controlled levels of ROS are required for modulating signaling networks that control physiological processes and stress responses (Miller *et al.* 2010).

1.3.2. Antioxidative defense system

To avoid oxidative damage during metal exposure, the antioxidative defense sytem is activated to maintain the increase of ROS within physiological concentrations (Sanità di Toppi and Gabbrielli 1999, Hall 2002, Guo *et al.* 2008). This defense system consists of both enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), and metabolites, such as glutathione (GSH) and ascorbate (AsA) (Arora *et al.* 2002, Halliwell 2006). The SODs constitute the first line of defense against ROS. They catalyze the dismutation of $O_2°^-$ to H_2O_2 and depending on the co-factor used, different SOD isoforms can be distinguished. Manganese SODs (MSD) are predominantly found in the mitochondrial matrix, while copper and zinc SODs (CSD) are located in the cytosol, chloroplast and extracellular space. The iron-containing SODs (FSD) are only found in the chloroplast (Halliwell 2006, Asada 2006). The H_2O_2 produced by SODs is scavenged and detoxified by CAT, mostly located in the peroxisomes, or by peroxidases (Halliwell 2006, Gechev *et al.* 2006). Plants are rich in peroxidases that remove H_2O_2 by using it to oxidize a co-substrate. In addition to ROS scavenging, they play a role in lignification, cross-linking of cell wall structural proteins and defense against pathogens (Halliwell 2006, Quan *et al.* 2008). Guaiacol peroxidase is a group of non-donor specific peroxidases in

plant cells, for which guaiacol is the common donor (Quan *et al.* 2008). They preferably oxidize aromatic electron donors to scavenge H_2O_2 in the cytosol, vacuole, cell wall and the extracellular space. In addition, they have a role in the biosynthesis of lignin (Sharma *et al.* 2012). Ascorbate peroxidases are important scavengers of H_2O_2 and use AsA as a reducing agent for the conversion of H_2O_2 to water (Conklin and Barth 2004, Smirnoff and Wheeler 2000), while GSH peroxidases use GSH. Besides enzymes, the metabolites GSH and AsA are low mass antioxidants that can scavenge ROS directly (Noctor *et al.* 1998, Smirnoff and Wheeler 2000, Conklin and Barth 2004, Meyer and Hell 2005). Glutathione plays a central role as chelating agent, antioxidant and signaling compound, as well as participates in the regulation of the cell cycle (Ball *et al.* 2004, Gomez *et al.* 2004, Verbruggen *et al.* 2009, Jozefczak *et al.* 2012), while AsA fulfills an essential role in physiological processes as well as in antioxidative responses (Davey *et al.* 2000, Pignocchi and Foyer 2003b, Foyer and Noctor 2005b, Hong-bo *et al.* 2008).

Enzymes and metabolites can cooperate to scavenge ROS. This is represented in the AsA-GSH cycle (Figure 1.3) where APX and AsA act together to detoxify H_2O_2 by the reducing power derived from NADPH. This cycle is located in various subcellular compartments. It further includes the successive oxidation and reduction of AsA and GSH with a cyclic transfer of reducing equivalents so that the plant-specific APX is able to reduce H_2O_2 to water. When H_2O_2 is scavenged by APX, AsA is oxidized to monodehydroascorbate (MDHA). The cellular pool of AsA is maintained in its reduced state by monodehydroascorbate reductase (MDHAR) using NADPH as reducing equivalent. Monodehydroascorbate can also spontaneously dismutate to dehydroascorbate (DHA), which in turn is reduced to AsA via dehydroascorbate reductase (DHAR) that uses GSH as electron donor. Oxidized GSH (GSSG) can be converted back to GSH by glutathione reductase (GR). The central role of GSH is the regeneration of AsA via the AsA-GSH cycle. Highly reduced GSH and AsA pools are essential for an optimal function of the AsA-GSH cycle (Mittler *et al.* 2004, Foyer and Noctor 2005a/b, Meyer 2008, Jozecfzak *et al.* 2012). The proper functioning of the AsA-GSH cycle is disturbed by oxidative stress resulting from metal exposure. Metal exposure also results in

a more oxidized DHA/AsA redox state, which affects cell division, cell wall biosynthesis, cell differentiation, senescence, etc.

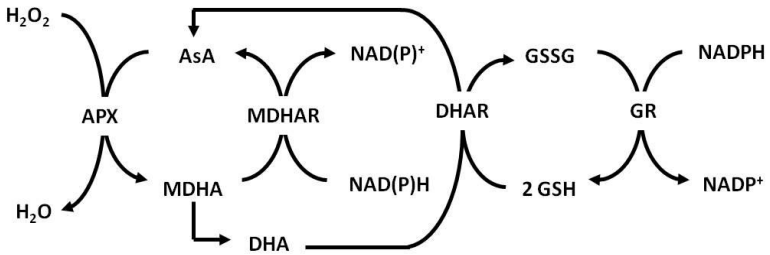

Figure 1.3. AsA-GSH cycle. This cycle plays an important role in the antioxidant defence mechanism to prevent ROS damage. Abbreviations: ascorbate (AsA), ascorbate peroxidase (APX), monodehydroascorbate (MDHA), dehydroascorbate (DHA), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), oxidised glutathione (GSSG), glutathione (GSH), glutathione reductase (GR).

The focus of this research will be placed on the AsA-related metabolism of the AsA-GSH cycle and its role in oxidative signaling during Cd or Cu stress will be studied. On the one hand, AsA is the metabolite that can scavenge ROS on its own, and on the other hand the enzyme APX can use AsA to scavenge H_2O_2. Ascorbate and APX are involved in many cellular processes. Metals may disturb AsA levels and/or redox state, or influence APX activity, thereby disturbing the cellular processes that these are involved in. In the following sections, an extensive review is presented, highlighting the diverse roles of AsA and AsA-related enzymes in cellular processes that are also influenced by metals. This framework of knowledge may be useful for revealing and understanding mechanisms related to AsA by which metals disturb cellular processes.

1.4. Ascorbate peroxidase

As mentioned above, controlled levels of ROS play an important role in modulating signaling networks that control both physiological processes and stress responses. Because H_2O_2 is a well-known example involved in the control of such processes and responses (Maksymiec 2007, Miller *et al.* 2010) and its production is an immediate response to increased Cd- or Cu-exposure, it might be a key molecule that can trigger signal transduction events after plant metal exposure (Mithöfer *et al.* 2004, Smeets *et al.* 2009). As a signaling molecule,

H_2O_2 can control cell proliferation and cell death to regulate plant growth and development, as well as the adaptation to abiotic stress factors and proper responses to pathogen attack (Quan *et al.* 2008, Miller *et al.* 2010). Important scavengers of H_2O_2 are CAT and APX. Moreover, APX has a higher substrate affinity for H_2O_2 in comparison to CAT, in the presence of AsA as a reductant. Therefore APX is able to detoxify low concentrations of H_2O_2 and as such is involved in the fine modulation of ROS for signaling (Mittler 2002). Thus, APXs are responsible for protection against harmful amounts of H_2O_2, as well as for the regulation of H_2O_2 levels for signal transduction (Rizhsky *et al.* 2002, Cuypers *et al.* 2011, Caverzan *et al.* 2012). Ascorbate peroxidases belong to the class I heme-peroxidases that play a key role in detoxification processes and are found in both algae and higher plants (Takeda *et al.* 1998, Takeda *et al.* 2000, De Gara 2004). The different isoforms of APX are classified according to their subcellular localization and are mainly present in chloroplast (thylakoid and stromal APX) and cytosol, but their presence in mitochondria, peroxisomes and the apoplast has also been reported (Arora *et al.* 2002, Mittler 2002, Gest *et al.* 2013).

To know whether APX isoenzymes are all involved in defense responses, a simultaneous analysis of the response of each APX isoenzyme in spinach leaves to several stress conditions has been carried out by Yoshimura *et al.* (2000). Four-week-old plants were exposed to high light intensity, salinity, drought, and treatmented with abscisic acid (ABA), methyl viologen and paraquat. Among the APX isoenzymes tested (chloroplast, cytosol and mitochondria), the transcript level of cytosolic APX (cAPX) increased in response to high light stress and paraquat treatment. The transcript levels of chloroplastidic and mitochondrial APX (chlAPX and mitAPX) did not change in response to any of these stresses, while the activities decreased as the intensity of light increased (Yoshimura *et al.* 2000). However, a wheat mutant (*Triticum aestivum*) showing decreased thylakoid APX (tAPX) activity had reduced photosynthetic activity when growing under high light intensity, suggesting that tAPX is essential for photosynthesis (Danna *et al.* 2003). Also, in *A. thaliana* plants lacking *tAPX*, an effect of photo-oxidative stress was observed showing increased H_2O_2 accumulation and oxidized proteins (Maruta *et al.* 2010). These results indicate that the genes for chlAPX are constitutively expressed for the immediate and efficient detoxification

of H_2O_2 under normal and stress conditions, while the gene expression of cAPX is responsive to environmental changes, resulting in the protection of important cellular compartments (chloroplasts,…) from oxidative stress and in strict control of the level of H_2O_2 in intracellular signaling (Yoshimura *et al.* 2000).

The two cytosolic isoforms in *A. thaliana*, APX1 and APX2, are the focal point of our study because they are stress responsive and differ in abundance. The transcripts of *APX1* are constitutively expressed in roots, leaves and stems, and they are significantly upregulated in response to diverse (a)biotic stress conditions. In contrast, the expression of *APX2* is almost undetectable in many plant tissues under normal conditions but is also significantly increased after exposure to (a)biotic stresses (Zimmermann *et al.* 2004, Frank *et al.* 2009).

The suggestion that APX1, although cytosolic, is also able to protect other organelles against oxidative stress (Yoshimura *et al.* 2000) was confirmed by Davletova *et al.* (2005). Moreover, APX1 was able to protect thylakoid, stromal and mitochondrial APXs during light stress, because a lack of APX1 resulted in degradation of these APXs and suppressed photosynthetic activity. Also, nuclear DNA during light stress was protected by APX1 (Davletova *et al.* 2005, Vanderauwera *et al.* 2011). Furthermore, Pnueli *et al.* (2003) showed that *APX1*-knockout plants are distinguished by an accumulation of H_2O_2 and a decrease in growth and development even under normal conditions, and that these plants have a higher sensitivity to light-induced oxidative stress. These results indicate that APX1 activity might be important in cross-compartment protection under oxidative stress generating conditions. Another cytosolic isoform, APX2, is only induced in the response of plants exposed to (a)biotic stress (Rossel *et al.* 2006, Koussevitzky *et al.* 2008). During heat stress, the expression of a cytosolic *APX* in tomato, homologous to *APX2* in *A. thaliana*, was significantly upregulated (Frank *et al*. 2009). In addition, an *APX2*-overexpressor showed enhanced tolerance to drought and high ABA levels in *A. thaliana* (Rossel *et al*. 2006), and to salt, drought and cold stresses in rice (Zhang *et al.* 2013). Furthermore, mutant plants lacking *APX2* had a decreased tolerance to light stress in *A. thaliana* plants (Suzuki *et al.* 2013) and to salt, drought and cold stresses in rice (Zhang *et al.* 2013). However *A. thaliana* lacking *APX2* also showed an increased tolerance to salt and paraquat-induced oxidative stress (Suzuki *et al.* 2013). Thus, a contrasting role for APX2 in the response to these

abiotic stresses was observed, which may depend on the plant species studied and the experimental conditions used.

From the above it is clear that *APX1* and *APX2* are involved in the acclimation of plants to abiotic stress conditions. Regarding metal stress, it has been demonstrated that APXs were involved in H_2O_2 scavenging and hence acting as regulators of signaling pathways under Cu and Cd stress (Lee *et al.* 2007a, Cuypers *et al.* 2011). The activity of APX was increased in different plant species exposed to Cu (Gupta *et al.* 1999, Cuypers *et al.* 2000, Tewari *et al.* 2006, Cuypers *et al.* 2011, Thounaojam *et al.* 2012). Also, an elevated APX activity was shown in many plants species after Cd exposure (Schützendübel *et al.* 2001, Aravind and Prasad 2005, Smeets *et al.* 2005, Lee *et al.* 2007a, Liu *et al.* 2007, Smeets *et al.* 2009, Cuypers *et al.* 2011). Furthermore, the transcripts of *APX1* were decreased in the roots and increased in the leaves of *A. thaliana* after 24 h Cu exposure (Smeets *et al.* 2009; Cuypers *et al.* 2011). In *A. thaliana*, an upregulation in the transcripts of *APX1* was observed in the roots after 24 h exposure to 10 µM $CdSO_4$ (Smeets *et al.* 2009) and in the leaves after 24 h exposure to 10 or 20 µM $CdSO_4$ (Smeets *et al.* 2008b, Smeets *et al.* 2009, Cuypers *et al.* 2011). The expression of *APX2* was increased in the roots of *A. thaliana* after 24 h exposure to 2 or 5 µM $CuSO_4$ (Cuypers *et al.* 2011) and after 24 h exposure to 5 and 10 µM $CdSO_4$ (Cuypers *et al.* 2011, Keunen *et al.* 2013). Moreover, upregulations in *APX2* were found in the leaves of *A. thaliana* after 24 h exposure to 5, 10 or 20 µM $CdSO_4$ (Smeets *et al.* 2008b, Smeets *et al.* 2009, Cuypers *et al.* 2011, Keunen *et al.* 2013). Although the changes in APX mRNA levels and/or activity under metal stress are indicative of a role of APX in metal stress responses, this has so far not been confirmed by studying downstream responses in *APX* mutants.

1.5. Ascorbate

Besides the function of AsA in the regulation of defense and survival of plants, it is also involved in modulating plant growth. L-ascorbic acid (ascorbate, AsA, vitamin C) is quantitatively the predominant antioxidant in plants. It is present in all subcellular compartments with an average concentration of 2 to 25 mM, or even higher in chloroplasts (Ishikawa and Shigeoka 2008, Linster *et al.* 2008).

Because of this high cellular content in plants, they are the main dietary source of vitamin C for humans (Linster *et al.* 2008, Valpuesta *et al.* 2004). Ascorbate is also an essential compound for the plant itself, with important roles as an antioxidant and as a modulator of plant development through hormone signaling (Valpuesta *et al.* 2004) and with essential roles in multiple physiological processes (Zhang 2013), some of which are disturbed by excess metals. In the following section, known direct effects of excess metals on AsA biosynthesis and functioning will be discussed, as well as possible interference of metals with the role of AsA in physiological and biochemical processes.

1.5.1. Ascorbate biosynthesis and the influence of metal exposure

1.5.1.1. Biosynthesis

Different pathways of AsA biosynthesis have evolved in animals and plants. In animals, AsA is formed from UDP-D-glucuronate to L-gulono-1,4-lactone via D-glucuronate formation, reduction and lactonization and via oxidation of L-gulono-1,4-lactone to L-ascorbic acid (Linster *et al.* 2007). In plants, the major biosynthetic pathway for AsA was discovered in 1998, and is known as the Smirnoff–Wheeler pathway (D-mannose/L-galactose pathway) (Figure 1.4) (Wheeler *et al.* 1998). This pathway involves the conversion of GDP-D-mannose to GDP-L-galactose. It has been suggested that L-galactose is a widespread constituent of plant cell walls (Smirnoff 2000a). Free L-galactose has never been measured in plants, but its rapid metabolism suggests that it may be present in low quantities. L-galactose is oxidized to L-galactono-1,4-lactone, which is the immediate precursor for AsA synthesis, by a NAD-dependent L-galactose dehydrogenase (GalDH). L-galactono-1,4-lactone is oxidized to AsA via L-galactono-1,4-lactone dehydrogenase (GalLDH), which is located at the inner mitochondrial membrane and donates electrons to cytochrome C (Smirnoff 2000a).

The initial three steps to D-mannose-6-P start with the conversion of D-glucose by hexokinases to D-glucose-6-P, which in turn is converted to D-fructose-6-P via phosphoglucose isomerase (PGI). The enzyme phosphomannose isomerase (PMI) that forms D-mannose-6-P has not been extensively studied in plants, although two putative genes are identified in *A. thaliana* (*At3g02570* and

At1g67070), based on sequence homology (Ishikawa *et al.* 2006). The activities of the next two enzymes, phosphomannomutase (PMM) and GDP-D-mannose pyrophosphorylase (GMP) in this pathway result in the formation of GDP-D-mannose (Linster *et al.* 2008). As with PMI, little is known about the PMM enzyme in plants, but based on sequence homology, *At2g45790* is a candidate *A. thaliana* PMM gene. Conklin *et al.* (1999) have presented evidence that the GMP enzyme, which catalyzes the synthesis of GDP-D-mannose from D-mannose-1-P, is encoded by the *VTC1* locus (At2g39770) in *A. thaliana*. The *vtc1-1* AsA-deficient mutant has a reduced conversion of mannose to AsA; and the activity of GMP is 30% in extracts from *vtc1-1* mutants as compared to wild-type plants (Conklin *et al.* 1999). These GDP-sugar intermediates are also involved in the synthesis of cell wall polysaccharides and glycoproteins (Smirnoff 2000b). The enzyme GDP-mannose 3',5'-epimerase (GME) is known to catalyze the conversion of GDP-D-mannose to GDP-L-galactose, which is then proposed to be broken down in two steps to L-galactose. Until recently, there was a missing link between GDP-L-galactose and L-galactose. Dowdle *et al.* (2007) and Laing *et al.* (2007) identified the enzyme converting GDP-L-galactose to L-galactose-1-P, known as GDP-L-galactose phosphorylase (GLGalPP). They have identified the gene *VTC2* (*At4g26850*) encoding this enzyme. They tested single mutants for this gene, *vtc2-1* and *vtc2-2*, which lack an active *VTC2* gene, but still contain 10%–20% of the wildtype AsA level. Therefore, other enzymes or pathways must synthesize the remaining AsA. They identified another *A. thaliana* gene, a homolog of *VTC2*, namely *VTC5* (At5g55120) encoding a second GLGalpp with similar properties to VTC2, and which is generally expressed at a 100–1000-fold lower than *VTC2*. The *vtc5-1* and *vtc5-2* mutants contain approximately 90% of the wild-type AsA level. They investigated their function further by constructing double mutants. Double mutants without functional *VTC2* and *VTC5* were unable to survive without AsA, showing that the GDP-mannose pathway, using GLGalPP, is the only physiologically significant source of AsA biosynthesis in *A. thaliana* seedlings (Dowdle *et al.* 2007, Laing *et al.* 2007). In addition, *VTC4* encodes a specific L-galactose-1-P phosphatase (GalPP) that contributes to the hydrolysis of L-galactose-1-P to L-galactose. The observation that *vtc4* knockout mutants are only partially deficient in AsA, as well as GalPP activity, suggests that *VTC4* is not the only gene encoding an enzyme catalyzing this reaction in *A. thaliana*

(Conklin *et al.* 2006). The VTC2, VTC5 and VTC4 enzymes may prove to be important regulatory steps given the rapid rate of L-galactose and L-galactono-l,4-lactone conversion to AsA. The pathway prior to L-galactose is located in the cytosol, but the last step, the oxidation of L-galactono-1,4-lactone to AsA, is located in the inner mitochondrial membrane (Figure 1.4) (Smirnoff 2000b).

The expression of some genes, determined by quantitative reverse transcription PCR, involved in AsA biosynthesis in bean (*Phaseolus vulgaris*) nodules was affected by stress conditions and particularly by Cd exposure (Loscos *et al.* 2008). After 26 days, the bean nodules were exposed to 100 μM $CdCl_2$ for 4 days (Table 1.1). The mRNA levels of *GMP*, *GME*, *GalDH* and *GalLDH*, except *GalPP*, declined in Cd-exposed nodules relative to the control. Moreover, no correlation existed between *GalLDH* mRNA levels, GalLDH activity and AsA content, as the latter two remained unaffected under Cd stress. The authors suggested that the GalLDH activity in nodules is posttranscriptionally regulated in response to stress conditions (Loscos *et al.* 2008). This is in contrast to the study of Tamaoki *et al.* (2003), where a correlation between the mRNA level of *GalLDH*, its activity and AsA content was indicated in young and mature *A. thaliana* leaves. Such correlation was not observed in the roots, where the activity of GalLDH and AsA level were low despite a high level of *GalLDH* transcripts. It was also suggested that the expression of *GalLDH* gene may be posttranscriptionally regulated in *A. thaliana* roots. To attain these results, total RNA was extracted from young (2-week-old) rosette leaves, mature (6-week-old) rosette leaves, inflorescence stems, flower buds, cauline leaves and roots. Furthermore, diurnal changes in AsA pool size and in the level of *GalLDH* expression were analyzed in the leaves of 2-week-old seedlings, where diurnal changes in *GalLDH* transcripts, GalLDH activity and AsA content showed similar patterns (Tamaoki *et al.* 2003). Thus, the content of AsA, the activity of GalLDH and the accumulation of *GalLDH* transcripts vary with plant species or tissues.

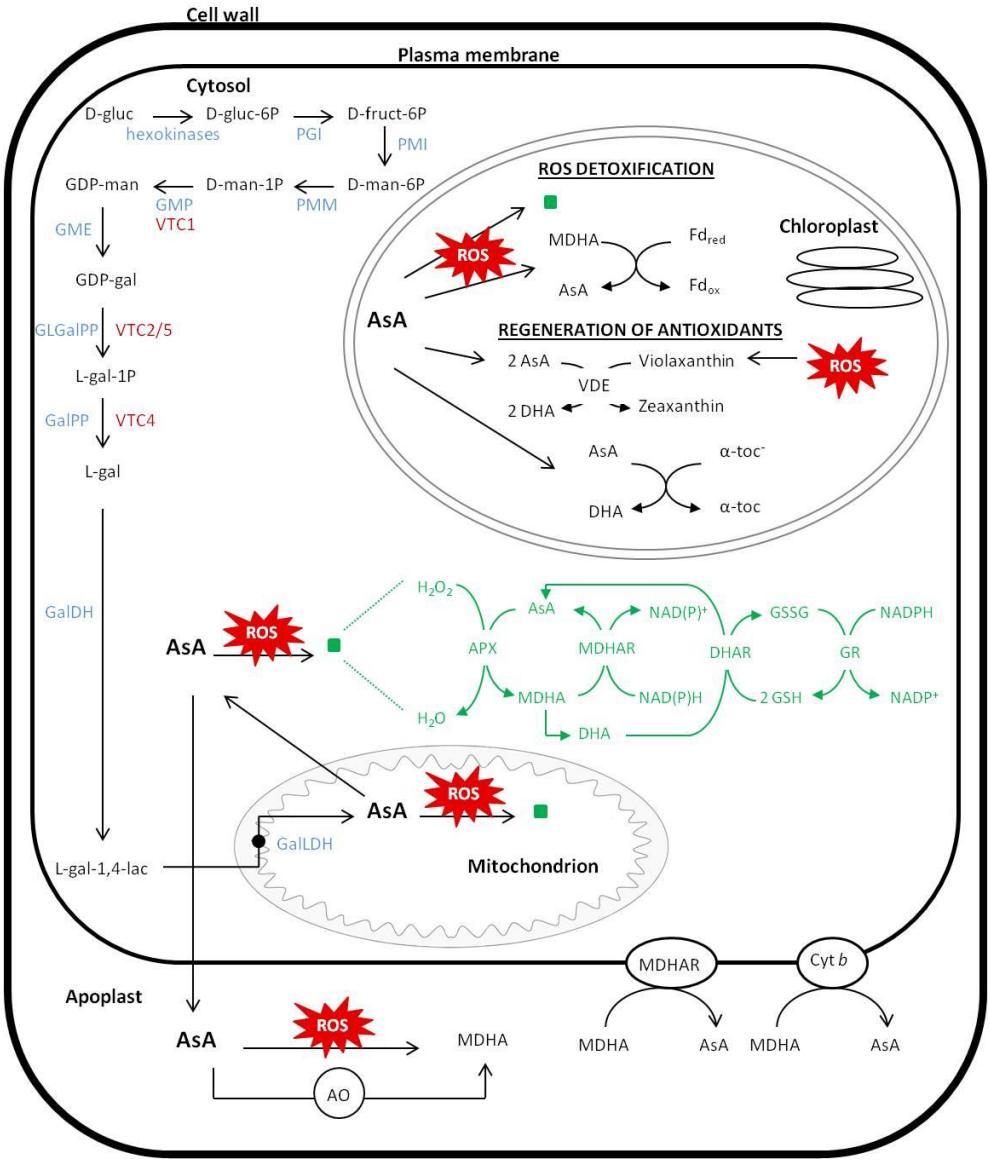

Figure 1.4. Representation of the biosynthesis, localization and antioxidant function of AsA. The biosynthesis of AsA takes place in the cytosol, except for the last step that occurs in the mitochondrion. Ascorbate plays a role in the antioxidant defense in two ways. One way is to scavenge ROS direct via the AsA-GSH cycle (■) or through direct binding to ROS and produce MDHA. The alternative is to regenerate antioxidants such as α-tocopherol and zeaxanthin.

Table 1.1. Exposure to excess metals has consequences for the biosynthesis of AsA and the antioxidant properties expressed as (1) the amount of reduced and oxidized ascorbate, *i.e.*, AsA and DHA and (2) the AsA-GSH cycle activities. The effects of excess Cd, Al, As, Pb, Cu, Zn, Ni and Mn are shown along with the experimental upset and the use of plant material. Abbrevations: AsA, ascorbate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase; APX, ascorbate peroxidase; GR, glutathione reductase, DHAR-OX, DHAR overexpressing, n.s., not significant.

	Plant	**Condition**	**Biosynthesis/content of total AsA**	**Antioxidant**		**Ref.**
				Ratios	**AsA-GSH cycle**	
NON-ESSENTIAL ELEMENTS						
Cadmium (Cd)	*Arabidopsis thaliana*	0, 5, 10 µM $CdSO_4$ 24 h	↑ Total AsA (roots)		↑ APX, GR (leaves)	Cuypers *et al.* (2011)
	Arabidopsis thaliana	0, 5, 10 µM $CdSO_4$ 24 h	↑ Total AsA (leaves)	↑ Reduced AsA (leaves) ↓ DHA/AsA (leaves)		Keunen *et al.* (2013)
	Arabidopsis thaliana	10 µM $CdSO_4$ 24 h	↑ Total AsA (roots)	↑ Reduced AsA (roots) ↓ DHA/AsA (n.s.)	↑ APX, GR (leaves)	Smeets *et al.* (2009)
	Phaseolus vulgaris (leaves)	2 µM $CdSO_4$ 0, 24, 48, 72 h	↑ Total AsA	Long term (>48 h) ↑ DHA Long term (>48 h) ↑ DHA/AsA	↑ APX, GR	Smeets *et al.* (2005)
	Phaseolus vulgaris	0, 100 µM $CdCl_2$ 4 days	↑ mRNA levels of GMP, GME, GalDH, GalLDH			Loscos *et al.* (2008)
	Pinus sylvestris	0, 5, 50 µM $CdSO_4$ 6, 12, 24, 48, 96 h	Transient ↑ total AsA followed by ↓ (50 µM Cd)	Transient ↑of reduced AsA followed by ↓(>24 h)	↑ MDHAR Transient ↓ APX followed by ↑ APX (>24 h)	Schützendübel *et al.* (2001)
	Ceratophyllum demersum	10 µM $CdCl_2$ 1 week	↓ Total AsA	↓ Reduced AsA DHA > reduced AsA ↑ DHA/AsA	↑ APX (2x) ↓ MDHAR, DHAR	Aravind and Prasad (2005)
	Brassica juncea (shoots)	0, 50, 200 µM $Cd(NO_3)_2$ 7 days	↓ Total AsA (200 µM Cd < controls)	↑ Reduced AsA (50 µM Cd) ↓ Reduced AsA (200 µM Cd < controls)		Mohamed *et al.* (2012)

Table 1.1. *Continued*

	Plant	Condition	Biosynthesis/content of total AsA	Antioxidant		Ref.
				Ratios	AsA-GSH cycle	
NON-ESSENTIAL ELEMENTS						
Cadmium (Cd)	*Brassica juncea* (roots)	0, 50, 200 μM $Cd(NO_3)_2$ 7 days	↑ Total AsA (50, 200 μM Cd) (50 μM > 200 μM > controls)	↑ Reduced AsA (50, 200 μM Cd) (50 μM > 200 μM > controls)		Mohamed *et al.* (2012)
	Bechmeria nivea Gaud	0, 1, 3, 7 mg/L Cd 1, 2, 3, 7, 10 days		↑ Reduced AsA followed by ↓ at high Cd concentrations ↑ DHA, DHA/AsA	↑ APX, GR	Liu *et al.* (2007)
	Shorea robusta	1 mg/L, 5mg/L and 10 mg/L $CdCl_2$ 4 months		↑ AsA		Pant *et al.* (2011)
Aluminum (Al)	*Oryza Sativa*	0, 80, 160 μM $Al_2(SO_4)_3$ 0, 5, 10, 15, 20 days	↓ Total AsA	↓Reduced AsA ↑DHA/AsA	↑ APX, MDHAR, DHAR, GR	Sharma and Dubey (2007)
	Nicotiana tabacum SR-1 wildtype	0, 400 μM $AlCl_3$ 24 h	↑ Total AsA	↑ DHA ↑ DHA/AsA	↑ APX	Yin *et al.* (2010)
	Nicotiana tabacum overexpressing *A. thaliana* cytosolic DHAR (DHAR-OX)	0, 400 μM $AlCl_3$ 24 h	↑ Total AsA	↑ Reduced AsA ↓ DHA ↓ DHA/AsA	↑ APX	Yin *et al.* (2010)
Arsenic (As)	*Shorea robusta*	1 mg/L, 5mg/L and 10 mg/L As_2O_3 4 months		↑ AsA		Pant *et al.* (2011)
Lead (Pb)	*Shorea robusta*	1 mg/L, 5mg/L, 10 mg/L $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ 4 months		↑ AsA		Pant *et al.* (2011)

Table 1.1. *Continued*

	Plant	Condition	Biosynthesis/content of total AsA	Antioxidant		Ref.
				Ratios	AsA-GSH cycle	
ESSENTIAL MICRONUTRIENTS						
Copper (Cu)	*Arabidopsis thaliana*	2, 5 µM $CuSO_4$ 24 h	↓ Total AsA (roots) ↑ Total AsA (leaves)	↑ DHA/AsA (roots)	↑ APX (roots) ↑ GR (leaves)	Cuypers *et al.* (2011)
	Arabidopsis thaliana	10 µM $CuSO_4$ 24 h	↓ Total AsA (roots) ↑ Total AsA (leaves)	↓ Reduced AsA, DHA (roots) ↑ Reduced AsA (leaves)	↓ APX (roots)	Smeets *et al.* (2009)
	Arabidopsis thaliana	0, 5, 25, 50, 100 µM $CuSO_4$ 1, 3, 7 days		Short term ↑ reduced AsA (1, 3 days) Short term ↑ DHA (3 days)	Short term ↑ DHAR Long term ↑ GR, MDHAR	Drazkiewicz *et al.* (2003)
	Phaseolus vulgaris (roots)	15 µM $CuSO_4$ 1, 5, 24, 48, 72, 96, 120, 168 h	↑ Total AsA	↑ Reduced AsA ↑ DHA ↑ DHA/AsA	Short term ↑ DHAR Long term slightly ↓ DHAR ↑ GR Long term ↑ MDHAR, APX	Gupta *et al.* (1999)
	Phaseolus vulgaris (leaves)	50 µM $CuSO_4$ 0, 24, 48, 72, 96, 120, 144, 168 h	↑ Total AsA	↑ Reduced AsA Short term ↓ DHA followed by transient ↑ DHA Long term ↓ DHA ↓ DHA/AsA	↑ MDHAR, GR Long term ↑ APX, DHAR	Cuypers *et al.* (2000)
	Oryza Sativa (MSE-9)	0, 10, 50, 100 µM $CuSO_4$ 1, 5 days	↑ Total AsA		↑ APX Long term ↑ GR (>50 µM Cu)	Thounaojam *et al.* (2012)
	Morus alba L. cv. Kanva-2	0.0, 0.1, 1.0, 100 µM $CuSO_4$ 25, 50 days	↑ Total AsA	↑ DHA (Cu-deficient,-excess) ↑ DHA/AsA (Cu-deficient,-excess)	↑ APX, GR	Tewari *et al.* (2006)
	Hordeum vulgare L. cv. Obzor	15, 150, 1500 µM $CuSO_4$ 5 days	↑ Total AsA	↓ % reduced AsA		Demirevska-Kepova *et al.* (2004)
Zinc (Zn)	*Phaseolus vulgaris* (roots)	50 µM $ZnSO_4$ 1, 5, 24, 48, 72, 96, 120, 144, 168 h	0-120h ↓ Total AsA >120h ↑ Total AsA	1–96 h ↓reduced AsA >96 h ↑ reduced AsA >72 h ↑ DHA ↑ DHA/AsA	1–96 h ↑ APX	Cuypers *et al.* (2001)

Table 1.1. *Continued*

	Plant	Condition	Biosynthesis/content of total AsA	Antioxidant		Ref.
				Ratios	AsA-GSH cycle	
ESSENTIAL MICRONUTRIENTS						
Zinc (Zn)	*Phaseolus vulgaris* (leaves)	50 µM $ZnSO_4$ 1, 5, 24, 48, 72, 96, 120, 144, 168 h	↑ Total AsA	↑ Reduced AsA 48 h, >96 h ↑ DHA >96 h ↑ DHA/AsA	↑ DHAR Long term ↑ MDHAR, APX, GR	Cuypers *et al.* (2001)
	Cajanus cajan LRG30 (long duration type)	2.5, 5.0, 7.5 mM $ZnSO_4 \cdot 7H_2O$ germinate for 6 days		↓ AsA	↑ GR	Rao and Sresty (2000)
	Cajanus cajan ICPL87 (short duration type)	2.5, 5.0, 7.5 mM $ZnSO_4 \cdot 7H_2O$ germinate for 6 days		↓ AsA	↑ GR	Rao and Sresty (2000)
	Brassica juncea	0.007, 0.05, 5.0, 10 mM ZnSO 10 days	↑ Total AsA		↑ MDHAR, DHAR, APX, GR	Prasad *et al.* (1999)
Nickel (Ni)	*Oryza sativa*	200, 400 µM $NiSO_4$ 0, 5, 10, 15, 20 days		↑ Reduced AsA ↑ DHA ↑ DHA/AsA	↑ MDHAR, APX, GR	Maheshwari and Dubey (2009)
	Cajanus cajan LRG30 (long duration type)	0.5, 1.0, 1.5 mM $NiSO_4 \cdot 6H_2O$ germinate for 6 days		↓ AsA	↑ GR	Rao and Sresty (2000)
	Cajanus cajan ICPL87 (short duration type)	0.5, 1.0, 1.5 mM $NiSO_4 \cdot 6H_2O$ germinate for 6 days		↓ AsA	↑ GR	Rao and Sresty (2000)
Manganese (Mn)	*Vigna unguiculata* cv. TVu 91	0.2, 50, 100 µM $MnSO_4$ 6 days		↓ Reduced AsA ↓ AsA/total AsA		Fecht-Christoffers *et al.* (2003)
	Vigna unguiculata cv. TVu1987	0.2, 50, 100 µM $MnSO_4$ 6 days			↑ MDHAR, GR	Fecht-Christoffers *et al.* (2003)
	Hordeum vulgare L. cv. Obzor	183, 1830, 18300 µM $MnCl_2$ 5 days	Transient ↑ total AsA followed by ↓		↓ APX	Demirevska-Kepova *et al.* (2004)
	Oryza sativa L. cv. Pant-12	3, 6 mM $MnCl_2$ 0, 10, 20 days		↓ Reduced AsA ↓ DHA ↑ DHA/AsA	↑ MDHAR, DHAR, GR, APX	Srivastava and Dubey (2011)

Current evidence suggests that the Man–Gal pathway predominates in AsA biosynthesis in plants, but it is plausible that an alternative pathway also exists (Wolucka and Van Montagu 2003, Valpuesta *et al.* 2004, Ishikawa and Shigeoka 2008, Linster *et al.* 2008). However, further studies are needed to clarify the role and existence of this alternative pathway in plants, as it remains to be fully proven.

1.5.1.2. Influence of Metal Exposure on AsA Biosynthesis

The biosynthesis of AsA in plants can be influenced by metals, depending on their properties, the duration of exposure, or the specific tissue of the plant studied (Table 1.1). A significant increase in total AsA content was observed in different plant species under various Cd conditions, probably due to a stimulation of AsA synthesis (Smeets *et al.* 2009, Cuypers *et al.* 2011, Smeets *et al.* 2005, Keunen *et al.* 2013). In 11-day-old *P. vulgaris*, supplied with 2 µM $CdSO_4$ to the roots during 3 days, a significant increase of the total AsA content was observed from 24 h until 72 h in the primary leaves of Cd-exposed plants (Smeets *et al.* 2005). In 3-week-old *A. thaliana* plants, treated with 5 or 10 µM $CdSO_4$ during 24 h, increases in total AsA were observed in roots (Smeets *et al.* 2009, Cuypers *et al.* 2011) and leaves (Keunen *et al.* 2013). These four studies demonstrated that, under short-term exposure of plants to the non-essential metal Cd, an increase in total AsA was observed.

Long-term exposure of Cd, similarly to exposure to higher concentrations, can lead to a decrease in total AsA, as shown in the studies of Schützendübel *et al.* (2001) and Aravind and Prasad (2005) (Table 1.1). In *Pinus sylvestris* (Schützendübel *et al.* 2001), Cd inhibits the antioxidative systems leading to H_2O_2 production, followed by a transient induction of the AsA synthesis. Five-week-old *P. sylvestris* plants were exposed to 5 or 50 µM $CdSO_4$ and root samples were collected after 6 to 96 h of exposure. In these trees, H_2O_2 accumulation was followed by a significant increase in total AsA after 12 h of exposure of the root tips to both Cd concentrations. From this point on, a depletion of total AsA was observed in root tips exposed to 50 µM Cd for a prolonged time (>12 h) (Schützendübel *et al.* 2001). Also, Aravind and Prasad (2005) reported a decrease in total AsA in *Ceratophyllum demersum* exposed to 10 µM $CdCl_2$ for 1 week (Aravind and Prasad 2005). Thus, Cd stress leads to a

decreased total AsA content after or during exposure to higher concentrations or longer exposure times.

In general, exposure of plants to excess levels of essential metals such as Cu and Zn, leads to an increase in total AsA content (Table 1.1). In a study of Tewari *et al.* (2006), mulberry (*Morus alba* L. cv. Kanva-2) plants were exposed for 25 or 50 days to 1 µM Cu used as control condition, 0 and 0.1 µM Cu (Cu-deficient conditions) and up to 100 µM Cu (excess supply). They showed that the amount of total AsA increased with increasing Cu concentrations, and Cu-deficient plants showed a decrease in the AsA content (Tewari *et al.* 2006). Also, other researchers observed a significantly higher total AsA content upon Cu stress. In barley (*Hordeum vulgare* L. cv. Obzor) leaves, this was noticed under severe Cu toxicity where plants were subjected to different concentrations of Cu [1.5 (control), 15, 150 or 1500 µM $CuSO_4$] (Demirevska-Kepova *et al.* 2004). In *P. vulgaris*, an increase in total AsA was observed in roots when exposed to 15 µM $CuSO_4$ from 1 h up to 1 week (Gupta *et al.* 1999), in leaves when treated with 50 µM $CuSO_4$ from 24 h up to one week (Cuypers *et al.* 2000), and in leaves when the beans were supplied with 50 µM $ZnSO_4$ from 1 h up to one week (Cuypers *et al.* 2001). Additionally, *A. thaliana* exposed for 24 h showed an elevated total AsA level in the leaves when exposed to 2, 5 (Cuypers *et al.* 2011), or 10 µM $CuSO_4$ (Smeets *et al.* 2009). This elevation was also observed in *Brassica juncea* exposed to 0.007, 0.05, 5, or 10 mM $ZnSO_4$ for 10 days (Prasad *et al.* 1999) and in rice (MSE-9) exposed to 0, 10, 50, or 100 µM $CuSO_4$ for 1 or 5 days (Thounaojam *et al.* 2012).

Although exposure of plants to excess Cu or Zn mostly led to increased total AsA content, a decrease in total AsA content was observed in the roots of *A. thaliana* plants exposed for 24 h to 2 and 5 µM $CuSO_4$ (Cuypers *et al.* 2011) or 10 µM $CuSO_4$ (Smeets *et al.* 2009). Similar results were reported for *P. vulgaris* roots supplied with 50 µM $ZnSO_4$ up to 96 h. However, when the exposure lasted beyond 96 h, an increase was observed (Cuypers *et al.* 2001). Besides Cu and Zn, Mn caused a transient increase in total AsA, followed by a decrease at high Mn exposure, which was observed in *H. vulgare* seedlings exposed to 183, 1830, or 18300 µM $MnCl_2$ (Table 1.1) (Demirevska-Kepova *et al.* 2004).

1.5.2. Interference of metals with physiological functions of AsA

1.5.2.1. Growth and development

The *vtc* mutants of *A. thaliana* and many transgenic plant species affected in AsA biosynthesis frequently show altered growth and development. To be viable, mutant plants must still contain at least a very low amount of AsA (for example: *vtc2*-mutant with 10% to 25% AsA of wild-type plants). Mutants without the metabolite AsA are lethal, which proves that it is a vital molecule for plant survival. Furthermore, there is evidence that AsA levels vary with plant developmental stages. Indeed, AsA, as well as its metabolic-related enzymes, are involved in the control of plant growth processes by controlling several basic biological processes, such as (1) the biosynthesis of hydroxyproline-rich proteins required for the progression of G1 and G2 phases of the cell, (2) the crosslinking of cell wall glycoproteins and other polymers, and (3) redox reactions at the plasmalemma involved in elongation mechanisms (Zhang 2013). When wild-type plants and *vtc-1* mutants were germinated on 1% (*w*/*v*) agar containing 1/4 Hoagland's nutrient solution, the *vtc-1* mutant showed modified shoot morphology and markedly decreased shoot biomass (Veljovic-Jovanovic *et al.* 2001). They also had smaller rosettes with approximately 50% of the wild-type rosette fresh weight after 5 weeks. Furthermore, the rosettes of the *vtc-1* mutant entered senescence earlier than those of the wildtype. The effect of the low AsA levels present in the *vtc-1* mutant could be due to the role of AsA in the plant cell cycle, or to the disruption of control mechanisms involved in cell division and/or elongation (Veljovic-Jovanovic *et al.* 2001).

Several reports showed that AsA is related with **cell division** in plants. Kerk *et al.* (1995) reported that AsA promoted the G1 to S progression in root meristems, whereas the non-dividing quiescent center had a high expression level of ascorbate oxidase (AO) and low AsA. Potters *et al.* (2000) showed that exogenously applied oxidized AsA and reduced AsA have a significant impact on the cell cycle progression and thus cell division or proliferation in tobacco suspension cells. In addition, *A. thaliana vtc* mutants, which have a low content of AsA, show delayed cell division, lowered growth rate of young branches and a slow plant growth (Tabata *et al.* 2001).

In general, the uptake of metals by plants cause growth inhibition that could be related to a decreased cell division which is attributable to disturbances in AsA redox balance. However, this remains to be proven. In wheat plants (*T. aestivum*) exposed to 1, 10 and 100 μM $CdCl_2$ during 48 h, ROS were detected in the root apex and the proliferation zone of the root apical meristem was reduced. The authors suggested that Cd-induced ROS production could affect G1 to S phase transition and progression through S phase (Pena *et al.* 2012). Maize seedlings exposed to 35 μM $Ni(NO_3)_2$, 10 μM $Pb(NO_3)_2$ or 3 mM $Sr(NO_3)_2$ showed a reduced mitotic index in the root cortex as well. Cell division was mainly inhibited by Ni, whereas Sr and Pb affected both cell division and elongation (Kozhevnikova *et al.* 2009). Zn excess also caused a strong decrease in root mitotic activity in *Saccharum spp.* (Radha *et al.* 2010).

Cell wall metabolism and cell growth are directly or indirectly affected by AsA, as well as by the enzyme AO (Davey *et al.* 2000). Ascorbate and its oxidation products (MDHA, DHA) influence plant cell expansion by a number of proposed mechanisms. One of these is the direct reaction of DHA with lysine and arginine side-chains to prevent crosslinking of cell wall proteins and polysaccharides, resulting in looser walls (Smirnoff 1996). DHA could also generate wall oxalate which might then influence free Ca levels through the formation of Ca oxalate crystals (Smirnoff 1996). In addition, the monovalent oxidation product, MDHA, has a role in regulating cell expansion as it is generated by AO activity and increases H^+-ATPase activity, which will then lead to increased cell expansion and solute uptake. Another mechanism is the direct scavenging of monolignol radicals involved in lignin biosynthesis by AsA, as well as the reversible inhibition of the cell wall/apoplastic peroxidases responsible for the formation of monolignol radicals (Davey *et al.* 2000). In addition, APX sustaining cell wall plasticity is found, by reducing the availability of H_2O_2 for other apoplastic peroxidase reactions and hence preventing lignification. Apoplastic peroxidases use H_2O_2 as an oxidant to produce monolignol radicals, a reaction inhibited by AsA that will scavenge these radicals. Thus, according to Davey *et al.* (2000), cell wall plasticity will be maintained due to the balance between AsA and H_2O_2 controlling the polymerization rate of lignin monomers. It is known that high AO

activity is correlated with areas of rapid cell expansion (Smirnoff and Wheeler 2000).

The enzymes involved in AsA biosynthesis can also regulate cellular processes. Torabinejad *et al.* (2009) reported that the enzyme VTC4 is a bifunctional enzyme that affects both myoinositol and AsA biosynthesis in plants. Myoinositol synthesis and catabolism are crucial for the production of phosphatidylinositol signaling molecules, glycerophosphoinositide membrane anchors and cell wall pectic non-cellulosic polysaccharides (Torabinejad *et al.* 2009). Another biosynthetic enzyme, GalDH, could have a role in cell expansion processes (Alhagdow *et al.* 2007). In plants with a reduced activity of GalDH, plant growth rate was decreased. The most affected plants, with 80% reduction in GalDH activity, showed a strong reduction in leaf and fruit size, mainly as a consequence of reduced cell expansion. All these results argue in favor of AsA and its accessory enzymes and oxidation products being closely related with the processes of cell wall metabolism and cell expansion (Alhagdow *et al.* 2007).

When different plant species are exposed to Cd, cell walls of roots and leaves are directly exposed to the metal excess. In different studies, a correlation between growth reduction and increased lignin content in roots has been considered as a typical event in defense against metal stress in different plant species (Cuypers *et al.* 2002, Chaoui *et al.* 2004, Lin *et al.* 2005, Yang *et al.* 2007, Kovácik *et al.* 2008, Finger-Teixeira *et al.* 2010, Tamás *et al.* 2010). Lignification can limit the cell expansion, the capacity for nutrient uptake and thus the ability to sustain plant growth (Schützendübel *et al.* 2001). In 3-day-old soybean seedlings supplied with 0, 25, 50, 75 or 100 μM $CdCl_2$ for 24 h, an inhibited root growth was observed, which was followed by and associated with lignin production and related parameters. The biosynthesis of lignin involves the polymerization of monolignols primarily derived from the phenylpropanoid pathway, which commences with the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL) to form cinnamate. The PAL activity increased after 50–100 μM Cd treatments, which strengthens the hypothesis that Cd induces lignification processes. Also, the cell wall-bound peroxidase activities significantly increased after 50–100 μM Cd exposure. These enzymes polymerize monolignols, which requires oxidative coupling and is dependent on H_2O_2. As an electron acceptor for cell wall-bound peroxidases,

H_2O_2 plays a major role in polymerization of phenolic monomers in the lignin biosynthesis. Content of H_2O_2 was increased when soybean seeds were exposed to 50–100 µM Cd, and the production of H_2O_2 has been correlated with the stiffening of cell walls as growth ceased and cells differentiated. Furthermore, the production of lignin was elevated, which stiffens the cell wall and restricts plant growth (Finger-Teixeira *et al.* 2010). In the roots of 21-day-old *Matricaria chamomilla* plants exposed to 60 or 120 µM $CdCl_2$ for 7 days, the activity of PAL was stimulated and accompanied with an increased content of lignin (Kovácik *et al.* 2008). Also in 5-day-old soybean seedlings treated with 0.2–1 mM $CdCl_2$, the lignin content significantly increased in the root tips. Moreover, the lignin biosynthesis related enzymes, peroxidases and laccases were enhanced during Cd treatment (Yang *et al.* 2007). Tamás *et al.* (2010) showed in barley seeds (*H. vulgare*) exposed to 1 mM $CdCl_2$ that stress activated several H_2O_2 generating enzymes, such as NADPH-oxidase, which probably contributes to general stress induced morphological changes of barley root tips (e.g. root thickening, cell wall modifications and lignification). These responses were accompanied by root growth inhibition due to the enhanced rigidification of cell walls and accelerated differentiation of root cells (Tamás *et al.* 2010). A high concentration of Cu also induced a high accumulation of lignin in the roots of 21-day-old *M. chamomilla* plants containing 60 or 120 µM $CuCl_2$ for 7 days (Kovácik *et al.* 2008). This is in accordance with the observation in soybean exposed to 1–10 mM $CuSO_4$ for 1–72 h, in which the lignin contents were significantly increased in the roots after 24 h (Lin *et al.* 2005). The aim of this early synthesis could be the immobilization of toxic metals in negatively charged sites of cell walls and restriction of their apoplastic transport (Lin *et al.* 2005). In a study by Chaoui *et al.* (2004), seeds of pea (*Pisum sativum*) were treated with 20 and 100 µM $Cd(NO_3)_2$ or 20 and 100 µM $CuSO_4$ for 4 days. The activities of lignifying peroxidases were not significantly altered, and NADPH oxidase activity was even inhibited during Cu treatment. In comparison to Cu, exposure to Cd stimulated the cell wall-lignifying peroxidases and increased the activity of NADPH oxidases. Microsomal APX activity, which was very low in the control, was markedly enhanced in metal-exposed plants. It is known that membrane-associated peroxidases are able to oxidize AsA and, consequently, could have an antioxidant role that seems to be stimulated in roots of Cd- and

Cu-treated pea. This is in addition to their contribution to the stimulation of the lignification process (Chaoui *et al.* 2004). In another study, the effect on guaiacol and syringaldazine peroxidases were investigated in roots and primary leaves of 11-day-old *P. vulgaris* seedlings exposed to 15 and 50 µM $CuSO_4$ and 50 µM $ZnSO_4$ (Cuypers *et al.* 2002). After exposure to 15 µM Cu, the capacity of guaiacol peroxidase increased from 24 h onward and the syringaldazine peroxidase activity rose significantly at 48 h in the roots of *P. vulgaris*. In contrast to Cu, a limited effect of Zn on the enzyme capacities was observed in the roots. In the primary leaves, it was observed that exposure to Cu or Zn resulted in increased capacities of both peroxidases. The function of these peroxidases in lignin biosynthesis is receiving more attention since many of the peroxidases studied are localized in the apoplast and play a key role in cell wall lignification. It is established that cell wall-associated peroxidases catalyze the final enzymatic step in lignin biosynthesis, *i.e.*, the oxidation of cinnamyl alcohols (Cuypers *et al.* 2002).

Senescence implies the coordinated degradation of macromolecules and the mobilization of regained nutrients, such as nitrogen, carbon and minerals, from senescing tissues to developing parts of the plant (Zimmermann *et al.* 2005). It is also characterized by a series of physiological and biochemical changes, such as chlorophyll degradation and a declining photosynthetic activity due to decreased expression of the Rubisco small subunit and chlorophyll a/b binding proteins. These genes are termed senescence-down-regulated genes (SDGs), while other genes are upregulated and therefore called senescence-associated genes (SAGs) (Barth *et al.* 2004, Zimmermann *et al.* 2005). In the later stages of senescence, cell peroxidation and DNA degradation occurs, which results in disintegrated organelles (Barth *et al.* 2004). It is well known that senescence is related to an increased level of free radicals, especially those derived from oxygen, as well as to a loss of antioxidant activity (Zimmermann *et al.* 2005).
Barth *et al.* (2004) showed that AsA influences senescence by modulating the expression of SAGs. The leaves of *vtc1* mutant plants lost chlorophyll more quickly and entered senescence earlier than the wild-type leaves. In addition, an upregulation of the expression of selected SAG transcripts was observed in the mutants, suggesting that AsA deficiency induces a senescent phenotype (Barth

et al. 2006). Due to its essential function as cofactor for enzymes involved in the biosynthesis of gibberellins, ABA and ethylene, AsA together with various phytohormones has a role in the senescence process. Abscisic acid and ethylene promote senescence, in contrast to gibberellic acid, which prevents senescence (Barth *et al.* 2006). Thus, the redox status of AsA may play a role in senescence by influencing complex phytohormone-mediated signaling networks, by modulating ROS accumulation and by stimulating the expression of SAGs.

Exposure to Cd is suggested to induce or accelerate leaf senescence. In senescent pea leaves, a role for the peroxisomal protease in the metabolic transition of leaf peroxisomes into glyoxysomes has been elucidated by Distefano *et al.* (1999). Pea (*P. sativum*) plants exposed to 50 µM $CdCl_2$ for 28 days were studied to determine the effect of Cd on the peroxisomal metabolism, and to find out whether these organelles are representative of the overall senescence symptoms promoted by Cd in leaves. The results showed that the peroxisome metabolism and proteolytic activity provide evidence for a Cd-induced senescence in pea leaves and suggest a role for peroxisomal proteases in the metabolic changes induced by metal stress (McCarthy *et al.* 2001). Furthermore, the Cd-induced changes observed by electron microscopy in the chloroplast structure showed the same pattern as that observed in other plant species treated with Cd, which are very similar to those found in senescent tissues. Taken together, these results indicate that Cd induces senescence symptoms in leaf peroxisomes (McCarthy *et al.* 2001). In tomato (*Lycopersicon esculentum*. Mill. cv. 63/5F1) treated with 50 µM $CdCl_2$ for 1 week, it was equally shown that Cd induces peroxisomal senescence in leaves by activating the glyoxylate-cycle enzymes malate synthase and isocitrate lyase, as well as peroxisomal peptidases, the latter being a well-known leaf senescence-associated factor (Chaffei *et al.* 2004).

1.5.2.2. Photosynthesis

Ascorbate is present in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall. Because of its central role in photosynthesis, the AsA concentration in chloroplasts can be as high as 50 mM as observed for spinach (Smirnoff 1996). Firstly, AsA protects the photosynthetic apparatus against ROS that are formed by oxygenic photoreduction in PSI (Mehler reaction) (Smirnoff 2000a). As CAT is

not present in chloroplasts, H_2O_2 is reduced by APX using AsA as reducing agent (Mazid *et al.* 2011). Secondly, AsA can directly scavenge $O_2°^-$, °OH and $^1O_2$ (Smirnoff 2000a). Thirdly, AsA contributes to the regeneration of α-tocopherol from α-tocopherol radicals that are formed during the reduction of lipid peroxyl radicals, thus protecting chloroplast membranes against oxidative degradation (Smirnoff 2000a). Furthermore, MDHA, the primary oxidation product of AsA, can act as a direct electron acceptor to PSI (Mazid *et al.* 2011). Finally, AsA is a co-factor for violaxanthin de-epoxidase (VDE), which is involved in photoprotection mediated by the xanthophyll cycle (Figure 1.4) (Smirnoff 1996, Arvidsson *et al.* 1996). The vital role of AsA in photosynthesis was demonstrated by transgenic rice plants with suppressed expression of GalLDH. These plants exhibited a loss of chlorophyll, a lower Rubisco protein content and a lower rate of CO_2 assimilation, leading to a slower plant growth rate and lower seed production (Liu *et al.* 2011).

Toxic metals generally influence the functions of the photosynthetic apparatus. They may interact with the photosynthetic apparatus at various levels of organization and architecture, such as in leaf tissues like stomata, mesophyll and bundle sheath cells, as well as with cytosolic enzymes (Prasad and Strzalka 2002). Metals may damage the electron transport activity by the induction of peroxidation and loss of thylakoid membrane integrity, thus altering the function of PSI and PSII (Prasad and Strzalka 2002, Kucera *et al.* 2008). The altered chloroplast structure and the substitution of Mn by Zn or Cd lead to the inactivation of the oxygen evolving complex, affecting the electron donation to PSII (Kucera *et al.* 2008). Finally, there is evidence that Cd has an influence on the activity of the chloroplasts by modifying chlorophyll content, which has been attributed to reduced chlorophyll synthesis or to enhanced enzymatic degradation (Ding *et al.* 2007, Anjum *et al.* 2008, Ekmekci *et al.* 2008, He *et al.* 2008, Mohamed *et al.* 2012). Ding *et al.* (2007) showed a significant loss of chlorophyll and carotenoid content in the leaves of *Alternanthera philoxeroides* exposed to 0.5, 1, 2, 5 and 10 μM $CdCl_2$ for 48 h, which worsened with increasing Cd concentrations (Ding *et al.* 2007). Similarly, in seeds of mustard (*Brassica campestris*) exposed to 0–100 mg/kg $CdCl_2$, the chlorophyll content was significantly decreased with increasing Cd concentration in the soil (Anjum *et al.* 2008). In another study, maize cultivars 32D99 and 3223, respectively

tolerant and sensitive to Cd exposure, were treated for 8 days to 0.3, 0.6 and 0.9 mM $Cd(NO_3)_2$. In this study, increasing Cd concentrations inhibited chlorophyll biosynthesis as well; the highest carotenoid content was observed in control plants for both cultivars, which gradually diminished with increasing Cd concentrations. Furthermore, it was shown that Cd inhibited the photoactivation of PSII. A decline in the parameter F_M, the maximum fluorescence in the dark-adapted state obtained by a saturation pulse (white light), suggests a change in the ultrastructure of thylakoid membrane, affecting the electron transport rate. The quantum efficiency of PSII (F_V/F_M) is often used as a stress indicator and describes the potential yield of the photochemical reaction and is decreased when exposed to increasing Cd concentrations for both cultivars (Ekmekci *et al.* 2008). Also in *Oryza sativa*, after 12 days of exposure to 50 µM $CdCl_2$, the F_V/F_M of Cd-sensitive mutant leaves was significantly lower than in the wildtypes. After 3 days of recuperation, F_V/F_M of the wild-type leaves was practically restored to the initial value, while F_V/F_M of the mutant was still significantly lower than that of the controls. The content of chlorophyll and carotenoids was decreased after exposure to Cd and the contents were lower in the leaves of the mutants than in the wildtypes (He *et al.* 2008). In *B. juncea* grown for 7 days in 0, 50 or 200 µM $Cd(NO_3)_2$, Cd negatively affected chlorophyll and carotenoid contents and activated the xantophyll cycle. The decreased β-carotene content observed in Cd-exposed leaves may therefore allow ROS accumulation in the photosynthetic apparatus, in its turn leading to oxidative degradation of chlorophylls, and destabilization of the structure of PSII. A significant increase in the de-epoxidation index of *B. juncea* induced upon Cd exposure suggests the need to protect the photosynthetic apparatus of Cd-exposed plants from photoinhibition (Mohamed *et al.* 2012).

In general, the effect of metals on plants is largely a strong and fast inhibition of growth processes of the above- and underground parts, as well as a reduced activity of the photosynthetic apparatus, often correlated with progressing senescence processes (Maksymiec 2007). It is evident that AsA is an important antioxidant in chloroplasts to protect the photosynthetic apparatus. Therefore, further research is needed to explore the interaction between metals, the physiological functions and AsA.

1.5.3. Metabolism of AsA

A number of products are metabolized from AsA, including L-tartrate and oxalate. The accumulation of tartrate is restricted to a handful of plants, while oxalate is widely distributed and appears predominantly as crystals of Ca oxalate (Debolt *et al.* 2007). Calcium oxalate synthesis in plant tissues could therefore be involved in the regulation of cellular Ca concentration (Smirnoff and Wheeler 2000, Debolt *et al.* 2007). The extent of accumulation of tartrate in plants remains unclear. It is known that the dominant organic acid in grape (*Vitis vinifera*) berries is tartrate. Berry tartrate is largely responsible for controlling the pH of the juice in winemaking. Addition of tartrate during vinification minimizes oxidative and microbial spoilage (Debolt *et al.* 2004).

The details of tartrate and oxalate formation vary between species. Whereas oxalate may be derived from photorespiratory intermediates such as glycolate (Smirnoff and Wheeler 2000, Debolt *et al.* 2007), its main source in various species is AsA (Smirnoff 2000b). To form oxalate, the AsA carbon skeleton is cleaved at the C2/C3 position and is formed with the carbon atoms 1 and 2. For some plant species, the carbon atoms 3 to 6 form L-threonic acid, which may be further oxidized to form tartrate. In the grape family, the formation of tartrate is performed by the C4/C5 cleavage (Debolt *et al.* 2007, Loewus 1999). The AsA cleavage pathways showed species-specific differences that remain unresolved.

The oxalate crystals can be used as a sequestration mechanism of toxic metals. The immobilization of excess Mn in these crystals, reported in earlier studies (El-Jaoual and Cox 1998, González and Lynch 1999), is supposed to be a detoxification mechanism. In the leaves of *P. vulgaris* (tolerant and susceptible genotypes to Mn stress) receiving 200 µM $MnSO_4$, a depletion of AsA was observed without any changes in its redox state, that was even more pronounced in the susceptible genotypes (González *et al.* 1998). Also another study reported that AsA levels were diminished without increasing the percentage of oxidized forms of AsA under the highest concentration of Mn exposure (Demirevska-Kepova *et al.* 2004). During this research, they used 5-day-old *H. vulgare* seedlings that were treated with 183, 1830, 18300 µM $MnCl_2$ over 5 days. The AsA depletion was suggested in both studies to be due to

an elevated synthesis of oxalate, at the expense of AsA, needed for the immobilization of Mn in oxalate crystals.

The use of oxalate as a detoxification mechanism has also been reported for other toxic metals. It was suggested that excess chromium (Cr) in rice plants is bound to undissolved or low-bioavailable compounds such as oxalate (Zeng *et al.* 2011) when two rice genotypes (*O. sativa* L. cv. Xiushui 113 and cv. Dan K5) were exposed to different amounts of $K_2Cr_2O_7$ (0, 10, 50 and 100 μM) during 10 days. In *Leersia hexandra*, treated with 0, 5, 30 and 60 mg/L $CrCl_3$, Cr was bound to oxalate in the leaves (Zhang *et al.* 2007). Also, in the leaves of *Eichhornia crassipes* exposed to Cr, it might be bound to oxalate ligands, while in the roots Cr was hydrated by water (Lytle *et al.* 1998). These results indicate that oxalate can be important in Cr accumulation and detoxification.

1.5.4. Localization of AsA and its biochemical reactions

1.5.4.1. Subcellular localization of AsA

Although mitochondria are the only compartments in *A. thaliana* in which the synthesis of AsA molecules can be completed, the highest concentration of AsA in non-stressed wild-type plants was detected in nuclei and the cytosol (Zechmann *et al.* 2011). This demonstrates that AsA must be transported via the cytosol into other cellular compartments. Ascorbate has an important function in the detoxification of ROS produced in chloroplasts under non-stress conditions, but especially under high light stress when the amount of AsA reaches similar levels as those observed in the cytosol (Zechmann *et al.* 2011). During high light conditions, increased levels of AsA were also observed in the vacuoles (Zechmann *et al.* 2011). H_2O_2 that diffuses into the vacuoles is detoxified, while AsA is oxidized to MDHA and DHA, which are then transported into the cytosol for reduction to AsA (Takahama 2004). The high levels of AsA in the nuclei, which are approximately similar to those in the cytosol, may be due to the nucleus being freely permeable to AsA. The possible roles of AsA in the nucleus of plant cells is still unclear (Zechmann *et al.* 2011), but it could be essential for the protection of DNA against oxidative modifications as demonstrated for mammalian cells (Green *et al.* 2006). In addition, up to 10% of the AsA content of the whole leaf is localized in the apoplast. A key function of

this apoplastic AsA is redox buffering, which protects the plasma membrane from oxidative damage (Pignocchi *et al.* 2003a).

1.5.4.2. Role of AsA as a cellular antioxidant

One of the major roles of AsA is being an antioxidant that protects metabolic processes against H_2O_2 and ROS (Hong-Bo *et al.* 2008). Ascorbate protects the plant from oxidative stress, and when taken up by the diet (vitamin C) it can also protect mammals from various chronic diseases that would otherwise result from oxidative stress (Zhang 2013). In plants, ROS are generated during aerobic cellular metabolism and photosynthesis, as well as by biotic and abiotic stresses such as drought, ultraviolet radiation, wounding, ozone and a range of pollutants (Suza *et al.* 2010, Zhang 2013). Ascorbate can directly react non-enzymatically with $O_2°^-$, H_2O_2 and $^1O_2$, generating MDHA and DHA (Smirnoff and Wheeler 2000, Conklin and Barth 2004). In addition, it can act as a secondary antioxidant by regenerating α-tocopherol or in the regeneration of zeaxanthin in the xanthophyll cycle (Hong-Bo *et al.* 2008). As a cofactor, AsA influences many enzyme activities through a synergistic cooperation (Foyer and Noctor 2003, Foyer and Noctor 2005a,b). One such important enzyme, APX, is essential for the detoxification/reduction of H_2O_2 to water and has a high specificity and affinity for AsA, which is used as electron donor for this reduction. As such, APX is connected to and forms an important component of the AsA-GSH cycle (Figure 1.1; section 1.3.2). As mentioned before, the obtained oxidized forms of AsA (DHA, MDHA) in this cycle are reduced back into AsA by the reducing power derived from NADPH. (Smirnoff and Wheeler 2000, Mittler *et al.* 2004, Asada 1999, Koussevitzky *et al.* 2008). Thus, cellular levels of reduced AsA that are able to function in ROS scavenging are determined by both, the rate of AsA synthesis and the rate of recycling oxidized AsA back to its reduced form via DHAR and MDHAR.

Recent evidence suggests that AsA plays a prominent role in protection of plants against several environmental stresses. Dehghan *et al.* (2011) showed that exogenous application of AsA via a pretreatment resulted in an improved germination percentage and growth of salt-exposed seedlings of soybean cultivars. They suggested that AsA can protect soybean seedlings against salt-induced oxidative stress through an increase in their antioxidative capacity

(Dehghan *et al.* 2011). Also for several other stressors, such as biotic stressors (Khan *et al.* 2011) and UB-V (Conklin *et al.* 1996), a protective function of AsA has been suggested. Here, we focus on studies suggesting a role for AsA in plants exposed to excess metals.

Cadmium is a **non-essential, non-redox active** metal and the effects of this metal on the AsA-GSH cycle have been examined in several studies (Table 1.1). During short-term (24 h) exposure of *A. thaliana* plants to 10 µM $CdSO_4$, an elevated level of reduced AsA was reported in roots (Smeets *et al.* 2009) and in leaves (Keunen *et al.* 2013). In contrast, a longer exposure time (>24 h) to Cd resulted in a decrease in the content of reduced AsA in *C. demersum* treated with 10 µM $CdCl_2$ during one week (Aravind and Prasad 2005). This kinetic trend of an increase followed by a decrease was also found in *P. sylvestris* treated with 0, 5 and 50 µM $CdSO_4$ during 6–96 h (Schützendübel *et al.* 2001). The initial (after 6 h) increase in the amount of reduced AsA in Cd-exposed roots was followed by a decrease or even a drop below the detection limit after 24 h of Cd stress. Furthermore, decreases in the redox state (DHA/AsA ratio) were observed in *A. thaliana* exposed to 10 µM $CdSO_4$ during 24 h in roots (Smeets *et al.* 2009) and leaves (Keunen *et al.* 2013), while increased DHA level and DHA/AsA ratios were reported in different plant species exposed to Cd stress for longer times (>24 h). These latter observations were shown in *Bechmeria nivea* Gaud exposed to 0, 1, 3 and 7 mg/L Cd during 1–10 days (Liu *et al.* 2007), in leaves of *P. vulgaris* treated with 2 µM $CdSO_4$ during 0–72 h (Smeets *et al.* 2005) and in *C. demersum* supplied with 10 µM $CdSO_4$ during 1 week (Aravind and Prasad 2005). These results indicate that initially stimulation of AsA biosynthesis occurs increasing the level of reduced AsA that can act as a primary antioxidant. Thereafter, AsA can concomitantly function as a cofactor of APX in the AsA-GSH cycle to enzymatically detoxify ROS. Together, usage of AsA as a direct antioxidant and as a cofactor for APX goes hand in hand with an enhanced AsA oxidation in Cd stress. Indeed the activities of APX and/or GR were generally elevated in different plant species exposed to various concentrations of Cd for short (<24 h) and long (>24 h) periods (Table 1.1). Interestingly, in *P. sylvestris* exposed to 0, 5 and 50 µM $CdSO_4$ during 6–96 h, APX activities were initially decreased after 6 h of exposure to 50 µM Cd, but had recovered

and were significantly increased after 24 h. This led to an accumulation of H_2O_2 after 6 h that was less pronounced after 24 h (Schützendübel *et al.* 2001). This transient rise in H_2O_2 levels may be important as a signal for the activation of cellular defenses, including APX (Prasad *et al.* 1994, Karpinski *et al.* 1999). In the same study, the redox state of AsA was also initially more oxidized in the presence of 50 µM Cd, as AsA was removing H_2O_2 non-enzymatically, and enhanced activities of MDHAR were reported (Schützendübel *et al.* 2001). In contrast, in *C. demersum* plants treated with 10 µM $CdSO_4$ during one week, reduction in the activities of MDHAR and DHAR were observed (Aravind and Prasad 2005). It has to be kept in mind that experimental set-up, *i.e.* Cd exposure concentrations and duration, as well as the plant species and their inherent uptake mechanisms, metal homeostasis and metal tolerance are important determinants in the outcome of Cd-induced oxidative stress responses. Therefore, generalization over different species and studies is hard to perform.

Aluminum is a non-essential redox active metal, which also decreases reduced AsA levels together with increased DHA/AsA ratios in roots and shoots of a rice cultivar of India Pant-12 exposed to 80 µM or 160 µM $Al_2(SO_4)_3$ during 0 to 20 days (Table 1.1) (Sharma and Dubey 2007). Nevertheless, plants tried to counteract this decline in reduced AsA by enhancing MDHAR and DHAR activities. In Al-exposed rice seedlings, these stimulations were more pronounced in the presence of 80 µM Al as compared to 160 µM Al. Furthermore, an increased H_2O_2 content was observed in these rice plants exposed to 160 µM Al, while a decline was shown at 80 µM Al exposure. The authors suggested that the generation of severe oxidative stress in rice plants due to Al treatment of 160 µM is responsible for the failing of the antioxidative defense system. The observed elevation in APX activity in rice seedlings may have been sufficient to remove H_2O_2 at 80 µM exposure, but the increased APX activity under 160 µM Al exposure may not have been sufficient, hence H_2O_2 levels increased (Sharma and Dubey 2007).

The regeneration of AsA was also studied in *Nicotiana tabacum* wild-type SR-1 plants and in transgenic tobacco plants overexpressing *A. thaliana* cytosolic DHAR (DHAR-OX) and MDHAR (MDHAR-OX) exposed to 400 µM $AlCl_3$ (Table 1.1) (Yin *et al.* 2010). Under Al exposure, the DHAR-OX plants showed higher levels

of reduced AsA and lower DHA contents and thus a lower DHA/AsA ratio than that in wild-type and MDHAR-OX plants. This indicates an increased efficiency of AsA regeneration by overexpressing DHAR, but not by overexpressing MDHAR. Furthermore, there was a higher increase in APX activity in DHAR-OX plants under Al treatment as compared to wild-types. These findings suggest that overexpression of DHAR can stimulate the reduction of AsA under Al treatment and as such contributes to Al tolerance in tobacco.

Copper is an **essential redox-active** metal, and, in excess, influences the content of AsA in the cell (Table 1.1). Similar to non-essential metals, Cu induces an increased DHA/AsA ratio in different plant species exposed to various concentrations of $CuSO_4$. This result was seen, for example, in a study of Tewari *et al.* (2006), where mulberry (*M. alba* L. cv. Kanva-2) plants were exposed for 25 or 50 days to 1 µM Cu used as control condition and up to 100 µM Cu (excess supply). Furthermore, other studies showed an increase in DHA/AsA; in the roots of *A. thaliana* seedlings exposed to 2 and 5 µM $CuSO_4$ during 24 h (Cuypers *et al.* 2011) and in roots of *P. vulgaris* treated with 15 µM $CuSO_4$ during 1 h up to 168 h (Gupta *et al.* 1999). Roots are in direct contact with the nutrient solution and, hence, with the applied Cu. Moreover, Cu is mainly retained in the roots (Cuypers *et al.* 2011) and as Cu easily performs monovalent oxidations, it can directly react with reduced AsA and hence stimulate its oxidation.
In contrast, in the leaves of *P. vulgaris* exposed to 50 µM $CuSO_4$ during 0 h up to 168 h, a decline of the DHA/AsA ratio was shown. Here, reduced AsA was always elevated and a decrease in DHA was shown after 48 h (Cuypers *et al.* 2000). In the leaves of *A. thaliana*, an increased AsA level was also observed after 24 h treatment of 10 µM $CuSO_4$, which was in contrast with the roots of *A. thaliana* with the same treatment (Smeets *et al.* 2009) (Table 1.1). In addition, an enhancement of the activities of APX and/or GR was reported in different plant species treated with various concentrations of Cu for short- (<24 h) and long-term (>24 h) exposures (Table 1.1). Furthermore, the activities of MDHAR and DHAR were increased in roots (Gupta *et al.* 1999) and leaves (Cuypers *et al.* 2000) of *P. vulgaris* exposed to, respectively, 15 µM and 50 µM $CuSO_4$, which resulted in an elevated AsA content for both plant organs, but in an increase of DHA in the roots and a decrease of DHA in the leaves. Elevated activities of APX

and GR, together with the alterations of the DHA/AsA ratio suggest an activation of AsA–GSH cycle that works efficiently for the leaves, but is insufficient to maintain the reducing potential of the system as indicated by the increased DHA/AsA ratios in the roots.

Zinc and Ni are both also essential metals, but in contrast to Cu, they are not redox active. An increased DHA/AsA ratio was observed in the roots and leaves of *P. vulgaris* after exposure to 50 µM $ZnSO_4$ during 1 h up to 168 h (Table 1.1) (Cuypers *et al.* 2001). In roots, already after 5 h the DHA/AsA was elevated due to the temporary decrease of reduced AsA. In the leaves, there was also an enhancement of the DHA/AsA ratio, but after longer exposure to Zn (96 h). In the roots of *P. vulgaris*, elevations in the contents of reduced AsA and DHA were observed after 96 h and 72 h of Zn treatment, respectively. The enhancement of DHA content was greater than the enhancement of reduced AsA, resulting in a higher DHA/AsA ratio. During germination (6 days) of pigeon pea seedlings (LRG30-long duration type and ICPL87-short duration type) supplied with 2.5, 5 and 7.5 mM Zn, a decrease of reduced AsA was shown, which is corresponding to the initial decrease in reduced AsA in the roots of *P. vulgaris* exposed to 50 µM $ZnSO_4$ (Rao and Sresty 2000). In both, leaves and roots of *P. vulgaris* exposed to 50 µM Zn (Cuypers *et al.* 2001) and in *B. juncea* exposed to 5 and 10 mM $ZnSO_4$ during 10 days (Prasad *et al.* 1999), the APX activity was enhanced. Elevated activity of GR was observed in pigeon pea seedlings supplied with 2.5, 5 and 7.5 mM Zn during 6 days (Rao and Sresty 2000), in *B. juncea* exposed to 5 and 10 mM $ZnSO_4$ during 10 days (Prasad *et al.* 1999) and in the leaves of *P. vulgaris* treated with 50 µM $ZnSO_4$ during 1 h up to 168 h, suggesting that the AsA–GSH cycle is activated but nevertheless insufficient to maintain the redox state.

The DHA/AsA ratio was also higher with increasing Ni concentration in rice plants (*O. sativa* L.) exposed to 200 µM and 400 µM $NiSO_4$ for up to 20 days (Table 1.1) (Maheshwari and Dubey 2009). Both, the amounts of AsA and DHA, were higher in rice plants treated with Ni. This does not correspond with the results of Rao and Sresty (2000), in which AsA content decreased in pigeon pea seedlings (LRG30-long duration type and ICPL87-short duration type) supplied with 0.5, 1 and 1.5 mM Ni during 6 days of germination. Additionally, the effect of excess Mn on AsA redox cycling has been examined (Table 1.1). A decline in reduced

AsA was shown in Mn-sensitive cowpea (*Vigna unguiculata*) cv. TVu 91 exposed to 50 and 100 µM $MnSO_4$ during 0, 10 and 20 days (Fecht-Christoffers *et al.* 2003) and in *O. sativa* L. plants exposed to 3 and 6 mM $MnCl_2$ during 6 days (Srivastava and Dubey 2011). In addition, the DHA/AsA ratio was increased in *O. sativa* L. plants exposed to 3 and 6 mM $MnCl_2$ during 6 days (Srivastava and Dubey 2011). In these plants treated with Mn, an increased activity in MDHAR, DHAR and GR was observed. These stimulated activities are supposed to induce AsA regeneration to maintain their requisite levels to inhibit the damage by Mn exposure. However, a decline in reduced AsA was shown, suggesting that enhanced activities of these enzymes were not sufficiently recovering reduced AsA from its oxidized forms (Srivastava and Dubey 2011). In contrast, Mn-tolerant cowpea cv. TVu 1987 exposed to 50 and 100 µM $MnSO_4$ showed an enhancement in the activities of AsA-regenerating enzymes like MDHAR and GR, and in these plants, no elevations of DHA were shown. This suggests that, in the Mn-tolerant genotype, the induction of enzymatic activity was sufficient, as opposed to the sensitive genotype.

General similarities involving the responses of AsA to metal stress could be observed. During short term (±24 h) plant exposure to Cd or Al, an increase in reduced AsA was reported, which coincided with a decrease in DHA/AsA. On the contrary, a decline of reduced AsA and/or an increase in DHA was shown during long term exposures along with an elevated ratio of DHA/AsA. With increasing time exposure and dose concentrations, this results in a shift to the oxidative side along with a decrease in efficiency of the antioxidative ability. A general trend towards an increased DHA/AsA ratio was also seen when plants were exposed to essential elements such as Cu and Zn. However, a distinction should be made between leaf and root responses. Whereas roots mainly demonstrated a decrease in reduced AsA, together with an increase in the ratio DHA/AsA, an increase of AsA was observed in leaves. The fact that the AsA pool in leaves remains reduced indicates an efficient AsA–GSH cycle, suggesting that signal molecules might be involved in the induction of this defense system against oxidative stress as a result of root metal uptake and/or translocation.

1.5.4.3. The interaction of AsA with the antioxidant α-tocopherol

In an indirect way, AsA is involved in ROS scavenging by regenerating α-tocopherol or by its role in zeaxanthin synthesis in the xanthophyll cycle (see section 3.2) (Hong-Bo *et al.* 2008). Alpha-tocopherol is the major vitamin E compound found in the membranes of the chloroplast envelope, thylakoid membranes and plastoglobuli (Sun *et al.* 2010). It is a lipophilic antioxidant that interacts with the polyunsaturated acyl groups of lipids to stabilize membranes, but is also able to quench various ROS and oxidized lipids (Hong-Bo *et al.* 2008). Tocopherols predominantly protect PUFAs from being oxidized (Hong-Bo *et al.* 2008) by preventing the propagation of lipid peroxidation, either by scavenging photosynthesis-derived ROS (mainly $O_2^{\bullet -}$ and OH°) or lipid peroxyl radicals in thylakoid membranes (Sun *et al.* 2010). While scavenging lipid peroxyl radicals, α-tocopherol itself is oxidized and can be reduced by reacting with AsA and other antioxidants (Hong-Bo *et al.* 2008). In general increased α-tocopherol levels contribute to plant stress tolerance. When 18-month-old rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and lemon balm (*Melissa officinalis*) were exposed to drought stress, α-tocopherol levels increased in the three species studied (Munné-Bosch and Alegre 2003). Exogenous AsA administration partly prevented α-tocopherol oxidation in osmotocially shocked chloroplasts in the light. Furthermore, chloroplastic α-tocopherol and AsA levels increased and no photo-inhibitory damage could be observed at relative leaf water contents between 58% and 86% in any of the species studied. These results suggest that a positive interplay exists between both AsA and α-tocopherol, where AsA may indirectly protect α-tocopherol by scavenging ROS and may participate in the recycling of α-tocopherol radicals (Munné-Bosch and Alegre 2003). In soybean seeds supplemented with 500 µM Fe-EDTA for 2 to 6 h, the content of α-tocopherol was not affected in the embryonic axes. However, after 24 h, significant increases in α-tocopherol concentration were observed during the initial steps of imbibition (Simontacchi *et al.* 1993). In 2-week-old *A. thaliana* plants exposed to either 5 or 50 µM $CdCl_2$, a significantly increased α-tocopherol content was able to prevent Cd-induced oxidative damage in chloroplasts (Sun *et al.* 2010). Furthermore, transgenic *B. juncea* plants over-expressing the γ-tocopherol methyl transferase gene had a six-fold increase in the level of α-tocopherol in comparison to the wild-type plants, and were used to test if the

increased α-tocopherol content would confer advantage to the plants exposed to Cd stress (Yusuf *et al.* 2010). They observed that the percentage germination of transgenic *B. juncea* seeds on medium supplemented with 20 mM $CdCl_2$ for 72 h was much higher (35.2%) than the germination of wild-type seeds under the same conditions (5.9%). Thus, the transgenic *B. juncea* plants had enhanced tolerance to the induced Cd stress, which was reflected in the photosynthetic performance (Yusuf *et al.* 2010). These studies showed that α-tocopherol is increased and needed when plants are exposed to Cd stress. Further research into the link between α-tocopherol and AsA under metal stress is needed and deserves special attention.

1.5.4.4. AsA oxidation by APX or AO serves differential functional goals in plant cells

Ascorbate peroxidase is an important AsA oxidizing enzyme using AsA as a reductant in the control of H_2O_2 levels in plant cells. Different APX isoforms, which exist in various cell compartments, are differentially responsive to the redox state (Smirnoff 2000b). Hydrogen peroxide can be formed by a two-electron reduction of O_2 at the level of the chloroplast and mitochondrial electron transport chains. Oxygen reduction by PSI and removal of the resulting H_2O_2 by APX contributes to the regulation of the redox state of photosynthetic electron carriers (Smirnoff 2000b). A study of Karpinski *et al.* (1997) showed that in 4-week-old *A. thaliana* exposed to a white light pulse, the transcripts of isoforms *APX1* and *APX2* (cytosolic isoforms) was rapidly and strongly increased.

Metals interfere with plant electron transport chains by enhancing electron leakage (Keunen *et al.* 2011a). These electrons can directly reduce O_2 leading to H_2O_2 production and many studies report increased APX activities as a result. Elevated APX activities were observed in Cd-exposed *C. demersum* (Aravind and Prasad 2005), *P. vulgaris* (Smeets *et al.* 2005), *B. nivea* gaud (Liu *et al.* 2007) and *A. thaliana* (Smeets *et al.* 2009, Cuypers *et al.* 2011). Furthermore, rice seedlings exposed to 160 µM $Al_2(SO_4)_3$ showed an increased H_2O_2 content, while a decline was shown during 80 µM Al exposure (Sharma and Dubey 2007). This suggests that plants exposed to the highest Al concentration suffer from severe oxidative stress and are unable to counteract through their antioxidative defense

system. Nonetheless, APX activity increased after exposure to both concentrations (Sharma and Dubey 2007). Tobacco plants overexpressing the *A. thaliana* cytosolic DHAR showed an increased APX activity after 24 h exposure to 400 µM $AlCl_3$ (Yin *et al.* 2010). In addition, excess levels of the essential metals Cu (Gupta *et al.* 1999, Cuypers *et al.* 2000, Tewari *et al.* 2006, Cuypers *et al.* 2011, Thounaojam *et al.* 2012), Zn (Prasad *et al.* 1999, Cuypers *et al.* 2001), Ni (Maheshwari and Dubey 2009) and Mn (Srivastava and Dubey 2011), resulted in enhanced APX activities.

Another enzyme catalyzing AsA oxidation is AO, which is mainly expressed in cell walls of fast growing plant cells (Smirnoff and Wheeler 2000). Although several studies unraveled biological functions for AO, its exact role still remains to be elucidated. Nonetheless, AO has been implicated as an apoplastically AsA oxidizer during cell elongation (Kato and Esaka 1999, Pignocchi *et al.* 2003b). In Tobacco BY-2 cells, DHA accumulation occurred after 4 days in the elongation culture, potentially caused by apoplastic AO (Kato and Esaka 1999). Pignocchi *et al.* (2003b) showed that enhanced AO activity positively affects plant growth with regard to both height and biomass. They also demonstrated that changes in apoplastic AO activity strongly affect the oxidation of apoplastic AsA contents without significant changes in whole leaf AsA content. While elevated AO activity leads to increased oxidation of the apoplastic AsA pool, decreased AO activity enhanced the amount of AsA as compared to DHA (Pignocchi *et al.* 2003b). The apoplastic redox state—regulated by AO activity—modulates plant growth and defense responses by regulating signal transduction cascades and gene expression patterns (Pignocchi *et al.* 2006). It was also suggested that metals can influence the redox state of the apoplast potentially by interfering with AO activity, which certainly deserves further investigation. Furthermore, Noctor and Foyer (2005a) and Fotopoulus *et al.* (2006) reported that AO could be a key regulator of the extracellular redox state and is able to alter the expression and activities of several AsA-related enzymes. There is clearly a great need to explore the role of the apoplastic AsA pool and AO even more, and not only in fast-growing cells but also in differentiating and lignifying tissues.

1.5.4.5. Other biochemical reactions

Ascorbate is an important cofactor of vital enzymes, thereby facilitating their catalyzed reactions. More specifically, it interacts with mono- or dioxygenases that contain Fe or Cu at their active site and require AsA to acquire maximal activity. The function of AsA is to maintain these transition metal ion centers in a reduced form (Davey *et al.* 2000). In addition, VDE also requires AsA to function in the photoprotective xanthophyll cycle (see section 3.2) (Zhang 2013). This cycle involves the conversion of violaxanthin to zeaxanthin in light-exposed leaves. Zeaxanthin is involved in non-photochemical quenching by dissipating excitation energy as heat (Smirnoff 2000a). Moreover, AsA acts as a prosthetic group for prolyl and lysyl hydroxylases, catalyzing the synthesis of hydroxyproline and hydroxylysine, respectively (Zhang 2013). Finally, AsA is shown to be a cofactor for different enzymes involved in the synthesis of ethylene, gibberellins and anthocyanins (Zhang 2013).

As demonstrated in several studies (Table 1.1), metal stress affects plant AsA contents. A change in the AsA redox state can also have an impact on the activities of enzymes that require AsA as a cofactor. Nonetheless, further research is needed to explore the connection between metal stress, AsA and its involvement in different biochemical reactions as described above.

1.6. Conclusions and mutants used in this study

The expression of cAPX enzymes is stress-inducible, and APXs are involved in both stress defense and fine-tuning of ROS levels for signaling. However, a potential role for APX and AsA in biological processes dealing with metal-induced oxidative stress and downstream responses in plants has not been studied. To that purpose a reverse genetics approach may be used to study Cd- and Cu-induced stress responses and signal transduction in knock-out *APX* mutants or mutants with reduced AsA levels.

The *A. thaliana APX1* knockout (*ko-apx1*) and *APX2* knockout (*ko-apx2*) plants are loss of function mutants that have a T-DNA insertion in the respective gene (Pnueli *et al.* 2003, Ecker 2003). Furthermore, two AsA deficient *A. thaliana* mutants have a defect in the AsA biosynthesis pathway. The *vtc1-1* mutant of *A. thaliana* was identified in a genetic screen for vitamin C (*vtc*) deficient mutants (Conklin *et al.* 1996), and has a cytosine to thymidine substitution in

the gene encoding the GDP-D-mannose pyrophosphorylase enzyme in the ascorbate synthesis pathway, leading to a substitution of the highly conserved proline into serine. As a result, the *vtc1-1* mutant has only 30% of the normal GMP activity and thus shows a reduced conversion of mannose to AsA, resulting in about 30% of wild-type AsA concentrations (Conklin *et al.* 1996). Another enzyme involved in the biosynthesis of AsA is GLGalPP, which occurs later in the ascorbate synthesis pathway, and is encoded by the *VTC2* gene. The *vtc2* mutant is a T-DNA-insertion mutant that contains 10%–20% of the wild-type AsA level (Conklin *et al.* 2000, Jander *et al.* 2002).

CHAPTER 2
Objectives

Anthropogenic activities have led to the distribution of toxic metals that often have a negative impact on the natural environment. Toxic metals in contaminated soils, such as cadmium (Cd) and copper (Cu), are able to accumulate in plants leading to great losses of crop production and plant diversity, and creates concerns for the safety of the food chain. After exposure to toxic concentrations of Cd and Cu, plants showed disturbances of morphological (growth reduction) as well as physiological (disruption of photosynthesis and respiration) mechanisms necessary for normal functioning. At the cellular level, Cd and Cu are known to produce reactive oxygen species (ROS) resulting in a disturbance of the cellular redox state, a condition known as oxidative stress. Although elevated levels of ROS are harmful and causing cellular damage, controlled levels of ROS play a role as signaling molecules in normal cell metabolism as well as in defense reactions. In plants, hydrogen peroxide (H_2O_2) has a role in oxidative signaling, controlling cell proliferation and cell death to regulate plant growth and development, as well as the adaptation to abiotic stress factors and proper responses to pathogen attacks (Quan *et al.* 2008, Miller *et al.* 2010). Because H_2O_2 is immediately produced after Cd or Cu exposure, it could be involved in signal transduction events triggered by metal stress. To protect plant cells from oxidative damage while still allowing ROS signaling, plants have evolved an antioxidative defense system consisting of enzymes and metabolites. Ascorbate peroxidase (APX) is an important scavenger with a high affinity for H_2O_2 providing the conversion of H_2O_2 to water using ascorbate (AsA) as reducing agent and is considered a potential regulator of the amount of H_2O_2 for signaling pathways. It was demonstrated that cytosolic APX (cAPX) is involved in defense responses, because it is responsive to environmental changes, resulting in protection of important cellular compartments from oxidative stress and in strict control of the level of H_2O_2 for intracellular signaling (Yoshimura *et al.* 2000). Ascorbate is a major redox buffer present in plants and can reduce ROS directly, or indirectly via APX. Ascorbate is

not only an important antioxidant but also has many other roles, e.g. it is a cofactor of many enzymes and a regulator of cell division and growth. Moreover, AsA can be involved in tuning cellular signaling pathways under (a)biotic environmental stress conditions because of its redox properties (Pignocchi and Foyer 2003, Foyer and Noctor 2005b). Since their involvement in controlling H_2O_2 levels, AsA and APX are involved in many cellular processes. Metals may disturb AsA levels and/or redox status, or influence APX activity, thereby disturbing the cellular processes that these are involved in. However, a potential role for APX and AsA in biological processes dealing with metal-induced oxidative stress and downstream responses in plants has not been studied.

The present research contributes to the knowledge of metal-induced oxidative stress and antioxidative defense in *Arabidopsis thaliana*. The main objective of this work was to study the involvement of cAPX and AsA in oxidative signaling and in the regulation of oxidative stress responses during Cu and Cd exposure, and to discover whether deficiency of APX and/or AsA could lead to an altered metal tolerance after short- or long-term metal exposure.

To reach these objectives three main experimental topics were followed:

1. To understand the role of cAPX in Cu- **(Chapter 4)** and Cd-induced **(Chapter 5)** oxidative signaling pathways and stress responses, knockout mutants of *APX1* (*ko-apx1*) and *APX2* (*ko-apx2*) in the Columbia-0 background were used. The influence of the mutation was measured using several indicators of the cellular redox state at morphological (DAB staining, root growth) and cellular level (gene expression, enzyme activities, metabolic measurements).
2. To study the importance of AsA in Cd- and Cu-induced stress responses and signal transduction, mutants with reduced AsA levels (*vtc1-1* and *vtc2*) were investigated for the expression of transcription factors and pro- and antioxidative enzymes that are regulated by metal-induced H_2O_2 signaling **(Chapter 6)**.
3. In the last part, the role of APX1, APX2 and AsA was examined in a long-term experiment to elucidate the relevance of the responses observed after short-term metal exposure, and to identify any

differences in tolerance to Cd or Cu between the four genotypes **(Chapter 7)**.

CHAPTER 3
Material and methods

3.1. Plant material and genotyping

Arabidopsis thaliana wild-type, *ko-apx1* (SALK_000249C), *ko-apx2* (SALK_018106C), *vtc1-1* and *vtc2* (SALK_146824C) mutant plants (ecotype Columbia-0) were used. *Ko-apx1* and *ko-apx2* mutants are both homozygous T-DNA insertion lines and were described in Pnueli *et al.* (2003) and Ecker (2003). Both *vtc* mutants are AsA deficient mutants. The *vtc1-1* mutant has a cytosine-to-thymidine substitution and was obtained in a screen of Col-0 EMS (ethyl methanesulfonate) mutants (Conklin *et al.* 1996). The *vtc2* plants are also homozygous T-DNA insertion lines (Conklin *et al.* 2000, Jander *et al.* 2002). All these mutants were obtained from Nottingham Arabidopsis Stock Centre (NASC, United Kingdom) and information about the mutants is summarized in Table 3.1.

Table 3.1. Details about the used mutants.

	ko-apx1	***ko-apx2***	***vtc2***	***vtc1-1***
Name	SALK_000249C	SALK_018106C	SALK_146824C	/
Mutation	T-DNA insertion line	T-DNA insertion line	T-DNA insertion line	Point mutation
Background	Columbia	Columbia	Columbia	Columbia
Genotype	homozygous	homozygous	homozygous	homozygous
Genotyping				
Forward primer (FP)	CCACCCTGGAA GAGAGGTTAG	ACCCGCTCATTT TTGACAAC	GTGTTCTTGACT GCTTGCCTC	TTGTTTTGTGTC GGTTGCAT
Reverse primer (RP)	CGATTTGAACA CATCCGTTG	GTTTCATTAACC CCTCCGGTA	GTTGCATTTGAA GCTTCTTGG	CCATTTGCTCTG TTTCGACA
T-DNA primer (LBb1.3)	ATTTTGCCGATT TCGGAAC	ATTTTGCCGATT TCGGAAC	ATTTTGCCGATT TCGGAAC	/
Size amplicon:				
FP-RP	No amplicon	No amplicon	No amplicon	± 500 bp
LBb1.3-RP	± 600 bp	± 600 bp	± 600 bp	/

All mutant plants were checked for homozygosity of the mutant allele. DNA was isolated and PCR amplification was performed using the Phire Plant Direct PCR Kit according to the manufacturer's instructions (Thermo Scientific, USA). Specific primers used are listed in table 3.1. The PCR program consisted of the following steps: 5 min at 98°C, followed by 40 cycles of 5 s at 98°C, 5 s at 60°C, 20s at 72°C, and a final step of 1 min at 72°C.

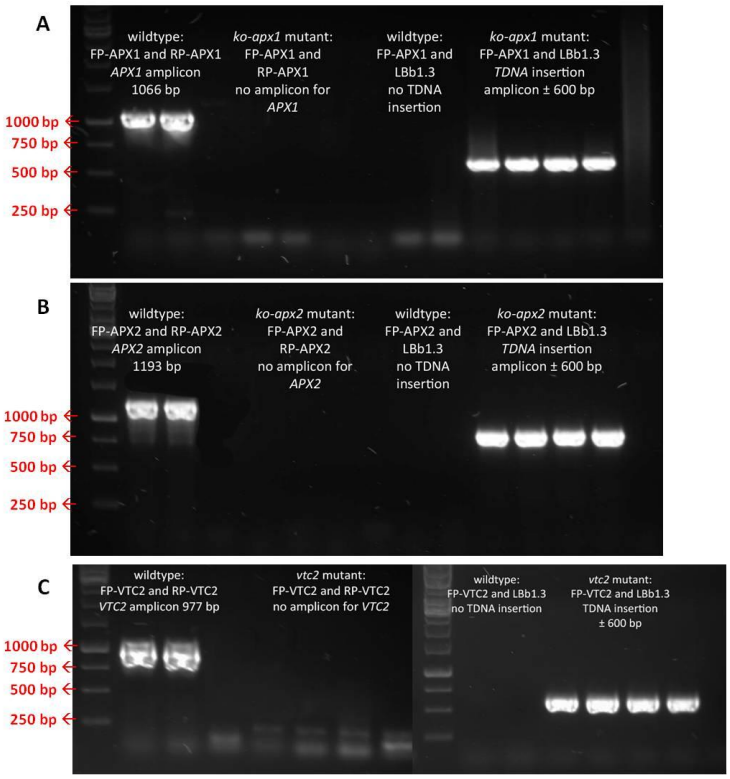

Figure 3.1. Genotyping of wildtype, *ko-apx1* (A), *ko-apx2* (B) and *vtc2* (C) plants using primers for the wild-type allele (top row) amplifying a 1066 bp (*APX1*), 1193 bp (*APX2*) and 977 bp (*VTC2*) fragment. These primers do not amplify the mutant allele as they bind at different sides of the T-DNA insertion and amplification of such a long fragment does not occur. A positive test for the mutant allele uses a T-DNA specific primer in combination with a primer in the *APX1*, *APX2* or *VTC2* gene (bottom row), yielding a PCR product of +/- 600 bp in the mutant and no PCR product for the wild-type allele. For wildtypes and mutants, respectively two and four biological repeats were used.

Amplified DNA was separated by 0.8% (w/v) agarose gel electrophoresis using 1X TAE (Tris-acetate-EDTA) buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and GelRed (Biotium, USA) nucleic acid stain. After running at 100 V for 1 h the PCR products and the 1 kb DNA ladder (Thermo Fisher Scientific, Belgium) were visualized via UV light in a GelDoc System (PowerPac™ Basic Power Supply, BioRad, Belgium). Results are given in Figure 3.1.

Because *vtc1-1* mutant has a point mutation, PCR fragments needed to be sequenced after excision from the gel and purification using the GeneJET Gel Extraction Kit (Thermo Scientific, Belgium). Samples were send to VIB Genetic Service Facility, University of Antwerp, for sequencing. Results are given in figure 3.2 and 3.3.

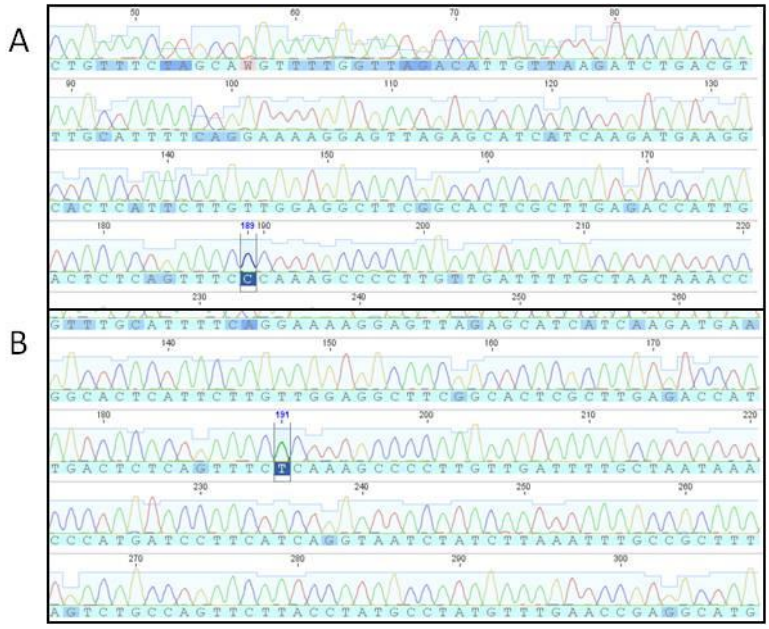

Figure 3.2. Sequencing wildtype and *vtc1-1*. A 'C' into 'T' point mutation is expected in the normal strand of *vtc1-1*. (A) DNA of wildtype amplified with FP-VTC1: coding strand of *VTC1* in wildtype gives a 'C' in position 189. (B) DNA of *vtc1-1* amplified with FP-VTC1: coding strand of *VTC1* in mutant gives a 'T' in position 191, corresponding to 189 in (A).

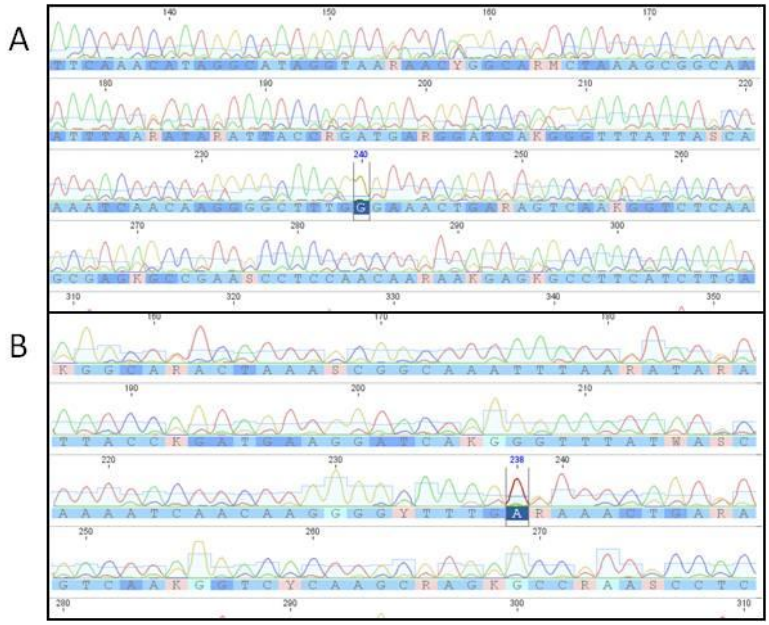

Figure 3.3. Sequencing wildtype and *vtc1-1*. A 'G' into 'A' point mutation is expected in the reverse complement strand of *vtc1-1*. (A) DNA of wildtype amplified with RP-VTC1: reverse complement strand of *VTC1* in wildtype gives a 'G' in position 240. (B) DNA of *vtc1-1* amplified with RP-VTC1: reverse complement strand of *VTC1* in mutant gives an 'A' in position 238, corresponding to 240 in (A).

3.2. Plant culture, treatment and growth analyses

Seedlings were grown in a diluted Hoagland hydroponic solution according to Smeets *et al.* (2008a), except that purified sand was used as a substrate instead of rock wool. The Hoagland solution contained macronutrients [0.505 mM KNO_3, 0.15 mM $Ca(NO_3)_2.4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.1 mM $MgSO_4.7H_2O$], micronutrients [4.63 µM H_3BO_3, 0.91 µM $MnCl_2.4H_2O$, 0.03 µM $CuSO_4.5H_2O$, 0.06 µM $H_2MoO_4.H_2O$, 0.16 µM $ZnSO_4.7H_2O$] and 1.64 µM $FeSO_4.7H_2O$ and 0.81 µM Na_2-EDTA. The hydroponic culture was placed in a climate chamber with a twelve hour photoperiod at 65% relative humidity and 22°C/18°C as day/night temperatures. Light was provided by a combination of blue, red and far-red LED modules (Green-Power LED modules, Philips, The Netherlands) with a light intensity of 170 µmol m^{-2} s^{-1} at the rosette level. During the first three days of germination, pots were covered with glass plates to prevent dehydration and to

obtain an even more controllable and stable growth situation. After 1 week of germination the nutrient solution was continuously aerated and since then the nutrient solution was refreshed every three days. After 19 days, the hydroponic solution was supplied with 2 µM $CuSO_4$ or 5 µM $CdSO_4$, similar to concentrations found in pore water of contaminated soils (Adriaensen *et al.* 2005, Krznaric *et al.* 2009) and plants were exposed for 0, 2, 24 or/and 72 h. During harvests, fresh weights of roots and leaves (entire rosette) were measured, samples were taken, snap frozen in liquid nitrogen and stored in a -70°C freezer for further analyses. A different sampling was used for element determinations and H_2O_2-staining as indicated in the specific sections.

To determine root growth parameters in the different genotypes, *A. thaliana* seedlings were grown on vertical agar plates on a modified 50-fold diluted Gamborg's B5 medium (12x12 cm petri dishes, Greiner Bio-one, The Netherlands), according to Remans *et al.* (2012), and contained macronutrients [0.5 mM KNO_3, 0.02 mM $MgSO_4$, 0.02 mM $CaCl_2$, 0.022 mM $NaH_2PO_4.2H_2O$, 0.02 mM $(NH_4)_2SO_4$], micronutrients [1.183 µM $MnSO_4.H_2O$, 0.090 µM KI, 0.97 µM H_3BO_3, 0.14 µM $ZnSO_4.7H_2O$, 0.1 µM $CuSO_4.5H_2O$, 0.021 µM $Na_2MoO_4.2H_2O$, 0.002 µM $CoCl_2.2H_2O$] and 2 µM $FeNO_3.9H_2O$. Seeds were surface sterilized, sown on the top of control plates [sucrose (5 g l^{-1}) was added to the medium, but no additional metals were applied], and after 2 days incubation at 4 °C in the dark vertically placed in the growth conditions described above. After 7 days, a homogenous subset of the seedlings was transferred to treatment plates (5 per plate) covering a concentration range of Cu (0-15 µM $CuSO_4$) or Cd (0-10 µM $CdSO_4$) and cultured for another 7 days. At the end of the experiment the vertical agar plates were scanned (Canoscan 4400F, USA) and root growth characteristics were measured using the Optimas image analysis software program (version 6.1, Optimas Corporation, USA) from which parameters were calculated in Excel.

3.3. Element determination

Roots and leaves were harvested to determine various element concentrations in both plant organs. To remove any Cu or Cd bound to the root surface, roots were washed with 10 mM $Pb(NO_3)_2$ at 4°C during 15 min and rinsed with dH_2O (Cuypers *et al.* 2002). Leaves were washed twice with dH_2O. Fresh weight was

determined (>200 mg), samples were dried for at least 48 h at 60°C, dry weight was determined, and samples were digested in 70% suprapur HNO_3 using a heat block. The concentration of Cd, Cu and other elements were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, 710 Series, Agilent Technologies, Belgium). In addition to the plant samples, blanks (HNO_3 only) and reference samples containing spinach (NIST Spinach (1570a)) were included.

3.4. H_2O_2-staining

Fresh leaves of *A. thaliana* plants were stained with 3,3'-diaminobenzidine (DAB) to visualize the presence of H_2O_2 using an adapted method of Thordal-Christensen *et al.* (1997). After harvesting, the leaves were placed in a 12-well plate either in 10 mM Na_2HPO_4-DAB solution (1 mg/ml DAB, 0.05% v/v Tween, pH 3.0) or in 10 mM Na_2HPO_4 (pH 7.0, control). The plates were all wrapped in aluminum foil and vacuum-infiltrated for 5 min using a desiccator. After incubation in a shaker (4 h, 80 rpm, room temperature, dark conditions), the 10 mM Na_2HPO_4-DAB and 10 mM Na_2HPO_4 solution were replaced with a bleaching solution (ethanol:acetic acid:glycerol 3:1:1) and incubated at 95°C during 15 min. The solution was refreshed and the plates were kept at 4°C. Finally, the samples were visualized with the BTV Pro software using a digital camera on a binocular microscope (Nikon, Belgium).

3.5. Determination of lipid peroxidation

The formation of thiobarbituric acid reactive metabolites (TBArm), like malondialdehyde (MDA, lipid oxidation end-product), is an indication of lipid peroxidation that was measured spectrophotometrically according to Dhinsda *et al.* (1981). Plant tissue was homogenized in 1ml of 0.1% trichloroacetic acid (TCA). After centrifugation (20 000 x g, 4°C, 10 min), the supernatant was diluted 3.5 times in 0.5% 2-thiobarbituric acid (TBA) in 20% TCA. The samples were incubated at 95°C during 30 min and rapidly cooled down on ice. After centrifugation (20 000 x g, 4°C, 10 min), the absorbance of the supernatant was measured at 532 nm and corrected for unspecific absorbance at 600 nm using UV-1600 spectrophotometer (Shimadzu, Germany).

3.6. Determination of AsA and GSH

Glutathione and AsA were measured according to a modified method by Queval and Noctor (2007) in the leaves of *A. thaliana*. Samples (50-100 mg) were ground in 200 mM HCl (5 µl/mg plant material) in a pre-cooled mortar. After centrifugation (20 000 x g, 4°C, 10 min), the pH of 300 µl extract was adjusted to 4.5 with 30 µl 200 mM NaH_2PO_4 (pH 5.6) and ca. 150 µl 200 mM NaOH. These aliquots were used to measure the reduced AsA content and the total GSH [GSH + glutathione disulfide (GSSG)] content or incubated with specific solutions prior to the analysis of total AsA [AsA + dehydroascorbate (DHA)] content and GSSG content.

Glutathione measurements relied on the glutathione reductase (GR)-dependent reduction of 5,5–dithiobis(2-nitro-benzoic acid) (DTNB) (600 µM) in the presence of NADPH (500 µM). To 10 µl of extract 190 µl of master mix [(2400 µl 200 mM NaH_2PO_4 - 10 mM EDTA (pH 7.5), 1440 µl dH_2O, 240 µl 10 mM NADPH, 240 µl 12 mM DTNB, 240 µl GR (20U/ml)] was added. In addition, a GSH standard (0 - 500 pmol GSH in 200 mM NaH_2PO_4) was integrated in the analysis. In order to measure GSSG, 140 µl extract was first incubated (30 min, 20°C) with 1% 2-vinylpyridine (2-VP) blocking any free reduced GSH. Similarly, 2-VP was also added to the GSSG standards to 1% (v/v) (0 - 100 pmol GSSG in 200 mM NaH_2PO_4). The aliquots were centrifuged twice (20 000 x g, 4°C, 10 min) to eliminate any residual 2-VP. To 20 µl supernatant 180 µl master mix [2400 µl 200 mM NaH_2PO_4 - 10 mM EDTA (pH 7.5), 1200 µl dH_2O, 240 µl 10 mM NADPH, 240 µl 12 mM DTNB, 240 µl GR (20U/ml)] was added. The change in absorption was immediately measured spectrophotometrically using a plate reader (FLUOstar Omega, BMG Labtech, Germany) at 412 nm during 5 min.

For total AsA analysis, an aliquot was incubated with 120 mM NaH_2PO_4 (pH 7.5) and 25 mM dithiothreitol (DTT) (1:1.4:0.1) during 15 min at 20°C, to convert DHA to AsA. Hereafter, the pH of the incubated supernatant was adjusted to 5.5 [the optimal pH for ascorbate oxidase (AO)] with 200 mM HCl. To measure the AsA content, 100 µl 200 mM NaH_2PO_4 (pH 5.6) and 55 µl dH_2O was added to 20 µl extract (reduced AsA) or 40 µl (total AsA). Total absorption was recorded at 265 nm using the FLUOstar Omega plate reader and after addition of AO [5 µl, (40 U/ml)], the oxidation of AsA was measured as a decrease of absorption at 265 nm after 15 min.

3.7. Analysis of enzyme capacities

Frozen root (150 mg) and leaf (200 mg) tissues were crushed under ice-cold conditions using a mortar and pestle. Sample powders were extracted in 2 ml extraction buffer (0.1 M Tris, 5 mM EDTA, 1% polyvinyl pyrolidone (PVP) (soluble), 5 mM 1,4-dithioerythritol (DTE), 1% nonidet P40, pH 7.8). The samples were placed in a shaker (30 min, 4°C), and centrifuged (50 000 x g, 4°C, 30 min) using corning tubes (Fisher Scientific, Belgium). Desalting of the proteins was obtained by $(NH_4)_2SO_4$ precipitation, in two subsequent steps of 40% and 80% respectively. Supernatants were incubated with the corresponding $(NH_4)_2SO_4$ concentration, placed in a shaker (30 min, 4°C) and centrifuged in corning tubes (50 000 x g, 4°C, 30 min). The pellet was dissolved in 1.75 ml Tris buffer (25 mM Tris in miliQ H_2O, pH 7.8), applied on top of a PD-10 desalting column (GE Healthcare, Belgium) and centrifuged (950 x g, 4°C, 2 min). The extract was stored in the -70°C freezer until the enzyme measurements were performed.

The enzyme capacity was measured spectrophotometrically (UV-1600 spectrophotometer, Shimadzu) under non-limiting reaction conditions at 25°C. Analysis of the activity of superoxide dismutase (SOD, EC 1.15.1.1) was based on the inhibition of cytochrome c at 550 nm (McCord and Fridovich, 1969). The $O_2°^-$ generating system consists of xanthine oxidase and xanthine. In the blanc cuvet, cytochrome C will be reduced by the formed $O_2°^-$. Addition of plant extract results in a disproportionation of $O_2°^-$ and hence an inhibition of the reduction of cytochrome C, which is a measure to define SOD activity. Scavenging of H_2O_2 was monitored by the activities of catalase (CAT, EC 1.11.1.6) and peroxidases (PX, EC 1.11.1.7). More specifically, CAT activity was measured via the reduction of H_2O_2 at 240 nm (Bergmeyer *et al.* 1974). Peroxidase activities were analyzed via the oxidation of different organic e^- donors, *i.e.* guaiacol (GPX) at 436 nm (Bergmeyer *et al.* 1974) or syringaldazine (SPX) at 530 nm (Imberty *et al.* 1984). NAD(P)H-depending enzymes were monitored at 340 nm according to Bergmeyer *et al.* (1974), and were the following enzymes GR (EC 1.6.4.2), malic enzyme (ME, EC 1.1.1.39), NADP-dependent isocitrate dehydrogenase (ICDH, EC 1.1.1.42),

glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12).

To extract the enzyme ascorbate peroxidase (APX, EC 1.11.1.11), a different extraction buffer (0.1 M Tris-HCl, 1 mM DTT, 1 mM EDTA, 10 mM ascorbate, pH 7.8) was used to homogenize the samples. After centrifugation (20 000 x g, 4°C, 10 min) the enzyme activity of APX was immediately measured at 298 nm at which AsA is oxidized in order to reduce H_2O_2, following the method of Gerbling *et al.* (1984).

The activities of all enzymes, except for SOD, were calculated according to the law of Lambert-Beer and corrected for the fresh weight used and the dilutions made during the procedure.

3.8. Analysis of gene expression

Frozen tissues (50-75 mg) were disrupted in 2 ml microcentrifuge tubes under frozen conditions using two stainless steel beads and the Retsch Mixer Mill MM 2000 (Retsch, Germany). Total RNA was isolated from the disrupted tissue using the Ambion RNAqueous Kit according to the manufacturer's instructions (Life Technologies, UK). The purity and the RNA yield were determined on the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA).

All RNA samples were adjusted to the same concentration before genomic DNA was eliminated using the Ambion TURBO DNA-free kit according to the manufacturer's instructions (Life technologies). The cDNA synthesis was performed using 1.2 µg total RNA and the high-capacity cDNA reverse transcription kit, making use of random hexamer priming, according to the manufacturer's instructions (Life Technologies) or the Primescript RT reagent kit (Perfect Real Time) using a combination of random hexamers and oligo(dT) primers, according to the manufacturer's instructions (Takara Bio Inc., Japan). A 10-fold dilution of the cDNA was made using 1/10 diluted Tris-EDTA buffer (1 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0) and stored at -20°C.

Quantitative real-time PCR was performed in an optical 96-well plate with an ABI-PRISM 7900 HT Real Time PCR System (Life Technologies) and the SYBR Green chemistry. Forward and reverse primers (Table 3.2) were designed via the Primer Express v2.0 software (Life Technologies) and ordered from Biolegio (The Netherlands). Specificity of the primers was checked in *silico* using TAIR

BLAST (http://www.arabidopsis.org) and after qPCR by verifying the occurrence of single peaks on the melting curve. For the location of primer binding see Table 3.2. The amplification efficiencies of all primer sets were investigated by a twofold serial dilution over six dilution points and were in between 85 and 115%, except *APX2* (80%). Information about the measured genes and used primers were collected in Table 3.2. The reactions were performed in a total reaction volume of 10 µl containing 2 µl of the diluted cDNA, 5 µl 2x Fast SYBR Green PCR Master Mix (Life Technologies), forward and reverse primers (300 nM each) in RNAse free H_2O. PCR amplification occurred at universal cycling conditions (20 s at 95°C, 40 cycles of 1 s at 95°C and 20 s at 60°C) followed by melting curve. The relative expression was calculated following the $2^{-\Delta Cq}$ method and was normalized to the expression of multiple reference genes (Vandesompele *et al.* 2002). Reference genes were chosen from Remans *et al.* (2008) and their expression stability was reconfirmed by the geNorm software (v3.5, qBase+, Biogazelle, Belgium, Vandesompele *et al.* 2012) and Normfinder algorithms (v0.953, Andersen *et al.* 2004). The reference genes used, as well as the method of RNA isolation and cDNA synthesis used in each experiment is summarized in Table 3.3.

3.9. Analysis of gene families

To calculate the relative abundance of gene family *CAT* and *MDHAR*, the expression level of each family member was determined for the control sample panel (wildtype, 24 h, 0 µM Cd) relative to the lowest expressed family member (*CAT1* and *MDHAR3*). This gives rise to a relative abundance factor for each member of the gene family, which is used in the calculation of its relative abundance in the time-course Cd exposure experimental setup. Data were normalized using the geometric average of the reference genes separately for roots and leaves; the most stable reference genes in both root and leaf control samples were determined by geNorm (Table 3.3). Subsequently, the changes in expression level for each member of a gene family were determined in function of the exposure time and Cd concentration applied and set relatively to the control (wildtype, 24 h, 0 µM Cd).

3.10. Statistical analysis

The obtained datasets of all measurements were analyzed using R (The R project for statistical computing; R Development Core Team 2008). Differences within a time point induced by treatment and between different genotypes were investigated using two way ANOVA and Tukey post-hoc test. Normal distribution of the data and homoscedasticity were checked using respectively the Shapiro test and the Bartlett test. If necessary the data was transformed [log(x), $\sqrt{x}$, e^x, 1/x] to achieve normality and homoscedasticity. When normality could not be reached, a non-parametric Kruskal-Wallis test was used followed by a Pairwise Wilcoxon rank sum test. Data of gene expression were all log-transformed prior to analysis.

Table 3.2. RT-qPCR primer information. Forward and reverse primers of all genes used for measuring gene expression, location of primer binding, amplicon size and the specificity of the primers are represented.

Gene	Locus	Annotation	Forward primer	Reverse primer	Exon location	Amplicon size	Primer efficiency (%)
Reference genes							
SAND family	AT2G28390	SAND family	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC	Exon 13	61 bp	107.80
F-box	AT5G15710	F-box protein	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA	Exon 1	63 bp	87.18
UBC	AT5G25760	ubiquitin conjugating enzyme	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC	E3-E4-jn	61 bp	98.70
TIP41-like	AT4G34270	TIP41-like	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA	E1-E2-jn	61 bp	91.21
expressed	AT4G26410	expressed	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC	E7-E8-jn	81 bp	90.54
EF-1α	AT5G60390	EF-1α	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA	Exon 1	76 bp	100.47
PPR	AT5G55840	PPR gene	AAGACAGTGAAGGTGCAACCTTACT	AGTTTTTGAGTTGTATTTGTCAGAGAAAG	Intron 2	59 bp	81.54
ACT2	AT3G18780	actin	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT	Exon 2	68 bp	88.05
Genes of interest							
Oxidative stress markers							
OX1	AT2G21640	mitochondrial oxidative stress marker UPOX	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC	Exon 1	91 bp	95.91
OX2	AT2G43510	defensin-like protein, marker transcript oxidative stress	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC	Exon 1	91 bp	96.84
OX3	AT1G19020	unknown protein, marker transcript oxidative stress	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA	Exon 1	92 bp	95.71
OX4	AT1G05340	unknown protein, marker transcript oxidative stress	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA	Exon 2	91 bp	99.10
OX5	AT1G57630	TIR, marker transcript oxidative stress	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC	Exon 1	91 bp	97.28

Genes coding for pro-oxidative enzymes							
RBOHC	AT5G51060	NADPH oxidase	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC	Exon 6	101 bp	92.31
RBOHD	AT5G47910	NADPH oxidase	AACTCTCCGCTGATTCCAACG	TGGTCAGCGAAGTCTTTAGATTCCT	Exon 1	96 bp	104.23
LOX1	AT1G55020	lipoxygenase	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC	Exon 6	101 bp	94.40
LOX2	AT3G45140	lipoxygenase	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC	Exon 3	102 bp	86.65
Genes coding for AsA or GSH biosynthesis genes							
GME	AT5G28840	GDP-mannose epimerase	GTTCACCGGCTCACGGAAAT	TGGGCTTCAGACCCGTTCTT	Exon 2	91 bp	93.76
VTC1	AT2G39770	GDP-mannose pyrofosforylase	TCTCAGTTTCCCAAAGCCCC	TTCATCAACTCCAACTGCCTTAAGA	Exon 1	91 bp	90.07
GSH1	AT4G23100	gamma-glutamylcysteine synthetase	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA	Exon 5	101 bp	98.60
GSH2	AT5G27380	glutathione synthetase	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC	Exon 11	101 bp	92.60
Genes coding for antioxidant enzymes							
CSD1	AT1G08830	Cu/Zn superoxide dismutase	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC	Exon 5	102 bp	93.80
CSD2	AT2G28190	Cu/Zn superoxide dismutase	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC	Exon 6	101 bp	93.90
Pri-MIR398a	AT2G03445	Primair micro RNA	AGAAGAAGAGAAGAACAACAGGAGGTG	ATTAGTAAGGTGAAAAAATGG	Exon 1	142 bp	96.45
Pri-MIR398b	AT5G14545	Primair micro RNA	AGTAATCAACGGCTGTAATGACGCTAC	TGACCTGAGAACACATGAAAACGAGAG	Exon 1	67 bp	85.29
Pri-MIR398c	AT5G14565	Primair micro RNA	TCGAAACTCAAACTGTAACAGTCC	ATTTGGTAAATGAATAGAAGCCACGGGCCACG	Exon 1	65 bp	104.18
FSD1	AT4G25100	Fe superoxide dismutase	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC	Exon 4	101 bp	88.80
MSD1	AT3G10920	Mn superoxide dismutase	ATGTTTGGGAGCACGCCTAC	AACCTCGCTTGCATATTTCCA	Exon 5	101 bp	89.40
CAT1	AT1G20630	catalase	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA	E5-E6-jn	103 bp	97.60
CAT2	AT4G35090	catalase	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG	Exon 2	76 bp	98.30
CAT3	AT1G20620	catalase	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA	Exon 2	91 bp	95.60
APX1	AT1G07890	ascorbate peroxidase	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA	Exon 5	101 bp	96.32
APX2	AT3G09640	ascorbate peroxidase	TTGCTGTTGAGATCACTGGAGGA	TGAGGCAGACGACCTTCAGG	Exon 3	91 bp	80.50
APX3	AT4G35000	ascorbate peroxidase	TTGAGCATCATAGGTTCCAGCA	CCGTGAGCTCCGTTCTCTCAT	Exon 8	91 bp	102.21
APX4	AT4G09010	ascorbate peroxidase	CTCGAAGTTGATTTGCTGTCCTAAG	CCGTGAAACCGTATCTCAGACTG	Exon 1	91 bp	95.24

Genes coding for antioxidant enzymes (*continued*)							
APX5	AT4G35970	ascorbate peroxidase	TGCAGTGGAGGTTACTGGCG	TGGATTTGGAAGTTCTCCATCGT	Exon 4	91 bp	90.69
APX6	AT4G32320	ascorbate peroxidase	CGGCCCAACAATTCCAGTAGT	CGAGGCACTCAGGGTTTCTG	Exon 6	91 bp	90.42
APX7	AT1G33660	ascorbate peroxidase	CCTTTTGTAGCATCAGGGAGACG	TCATTTCCACCCTTGAAGAGAGG	Exon 1	91 bp	90.34
sAPX	AT4G08390	stromal ascorbate peroxidase	GGACACCAGAGTGGCTGAAGTT	GCATCAGTGGGTAGGACAAGGAG	Exon 7	91 bp	94.32
tAPX	AT1G77490	thylakoid ascorbate peroxidase	ATTCTCTTGGAAGCGTCGCAT	CTCGTGCTCGTCCCATTGAT	Exon 1	91 bp	95.92
DHAR1	AT5G16710	DHA reductase	CCAGATTCACTTCCTTTCGTCAA	TTACATCCTCTGTTTCCGCCC	Exon 5	91 bp	94.00
DHAR2	AT1G75270	DHA reductase	ATCAGATGGGTCTTGTAGGGAAGC	GTGCTCCTGATGTTCTCGGC	Exon 2	91 bp	95.04
DHAR3	AT1G19550	DHA reductase	AACTCTTTCCCCGGCGATAA	CTGAATTTGCCTCTGTTGGCTC	Exon 1	92 bp	95.23
MDHAR1	AT3G52880	MDHA reductase	TGCACCAACCCCAACTATCAC	CACAGCTCAGCCAAATGGAGAG	Exon 3	91 bp	93.62
MDHAR2	AT5G03630	MDHA reductase	CCCAATTCCAGCAGCAACAT	TCCATATGAGCGTCCTGCACT	Exon 7	91 bp	93.68
MDHAR3	AT3G09940	MDHA reductase	AAATGATTGCGAGTTCACCAGG	TACGTGATCATTGGAGGTGGTGT	Exon 8	91 bp	96.62
MDHAR4	AT3G27820	MDHA reductase	GGTTACGCAGCTCTCGAATTCA	AGGCCTCTCATACGGTGCAA	Exon 1	93 bp	97.55
MDHAR5	AT1G63940	MDHA reductase	TAGTAGAAAGCGGATCGCCTGA	GCGCTTGCGAGTTTAGCCTT	Exon 16	91 bp	95.35
GR1	AT3G24170	glutathion reductase	CTCAAGTGTGGAGCAACCAAAG	ATGCGTCTGGTCACACTGC	Exon 15	101 bp	94.76
GR2	AT3G54660	glutathion reductase	GCCCAGATGGATGGAACAGAT	TAGGGTTGGAGAATGTTGGCG	Exon 5	91 bp	96.44
Genes coding for regulatory enzymes							
OXI1	AT3G25250	oxidative signal inducible	CGATTATTGTCCGGGACAGA	CTAATACAAGCTCCGCCGC	Exon 1	104 bp	104.40
MPK3	AT3G45640	map kinase	GACGTTTGACCCCAACAGAA	TGGCTTTTGACAGATTGGCTC	Exon 5	103 bp	100.37
MPK4	AT4G01370	map kinase	ACATGTCGGCTGGTGCAGT	AATATGGGTGGCACAACGC	Exon 5	96 bp	100.20
MPK6	AT2G43790	map kinase	TAAGTTCCCGACAGTGCATCC	GATGGGCCAATGCGTCTAA	Exon 5	101 bp	107.68
WRKY22	AT4G01250	transcriptiefactor	AAACCCATCAAAGGTTCACCA	GGGTCGGATCTATTTCGCTC	Exon 2	101 bp	101.59
WRKY25	AT4G01250	transcriptiefactor	AAACCCATCAAAGGTTCACCA	GGGTCGGATCTATTTCGCTC	Exon 2	101 bp	101.59
ZAT7	AT3G46090	zinc-finger transcription factor	TCACATGAGGAGACATAGGAACGAG	TCTTCAAAGCCGTCACCGTC	Exon 2	70 bp	99.06
ZAT10	AT1G27730	zinc-finger transcription factor	CTGGAAGTGGGAAATCACACG	TTCGTAGTGGCACCGCTTGT	Exon 1	92 bp	94.05
ZAT12	AT5G59820	zinc-finger transcription factor	GTGCGAGTCACAAGAAGCCTAACA	GCGACGACGTTTTCACCTTCTTCA	Exon 1	72 bp	98.00
HSF21	AT4G18880	heatshock factor	GAGTTTTCTAGAGATCTTCTTCCGAGATTC	TCCCATTGCTCAGGATCAGC	Exon 1	110 bp	83.15

Genes involved in the ethylene biosynthesis/signaling							
ACS2	AT1G01480	ACC Synthase	CATGTTCTGCCTTGCGGATC	ACCTGTCCGCCACCTCAAGT	Exon 3	91 bp	94.05
ACS6	AT4G11280	ACC Synthase	TTAGCTAATCCCGGCGATGG	ACAAGATTCACTCCGGTTCTCCA	Exon 3	92 bp	102.40
ERF1	AT3G23240	ethylene response factor	TCCTCGGCGATTCTCAATTTT	CAACCGGAGAACAACCATCCT	Exon 1	91 bp	95.77

Table 3.3. Summary of the method of RNA isolation and cDNA synthesis as well as the reference genes used in each experiment

Experiment	Chapter	RNA isolation	cDNA synthesis	Reference genes roots	Reference genes leaves
ko-apx | Cu	4	RNAqueous Kit	TURBO DNA-free kit Hihgh capacity cDNA reverse transcription kit	AT2G28390 AT5G15710 AT4G26410 AT5G25760 AT4G34270	AT2G28390 AT5G15710 AT5G25760 AT4G34270
ko-apx | Cd	5	RNAqueous Kit	TURBO DNA-free kit Primescript RT reagent kit (Takara)	AT4G34270 AT5G55840 AT3G18780	AT2G28390 AT5G15710 AT5G25760 AT4G34270 AT5G60390
vtc1-1 | Cd/Cu	6	RNAqueous Kit	TURBO DNA-free kit Hihgh capacity cDNA reverse transcription kit	AT2G28390 AT5G15710 AT5G60390	AT4G26410 AT2G28390 AT5G15710 AT5G60390
vtc1-1 and *vtc2* | Cd	6	RNAqueous Kit	TURBO DNA-free kit Primescript RT reagent kit (Takara)	AT4G34270 AT2G28390 AT5G15710 AT5G25760 AT5G60390	AT4G34270 AT3G18780 AT5G55840

CHAPTER 4

APX1 and APX2 are involved in Cu-induced oxidative signaling leading to growth inhibition in *Arabidopsis thaliana*

An Bielen, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2014). APX1 and APX2 are involved in Cu-induced oxidative signaling leading to growth inhibition in *Arabidopsis thaliana*. In preparation.

Abstract

Copper (Cu), locally present in the environment in elevated concentrations, induces oxidative stress in plants. Although reactive oxygen species (ROS) can cause irreversible damage to cellular components, they can also act as signaling molecules in the cell. ROS signaling in plants controls processes involved in normal metabolism as well as stress-related responses. Hydrogen peroxide (H_2O_2) is a well-known signaling molecule induced during metal stress and is scavenged by ascorbate peroxidases (APXs). Therefore, APXs could be involved in Cu-induced oxidative signaling. To unravel the role of APXs in this process, wild-type and 2 cytosolic *apx* knockout *Arabidopsis thaliana* seedlings were hydroponically grown and exposed to 2 µM Cu during 2, 24 and 72 h. Although *apx* knockout seedlings were observed to be more tolerant to Cu exposure than wildtypes, they experienced similar membrane damage. In roots, it was demonstrated that APX1 and APX2 are involved in Cu-induced RBOHD signaling, which in turn might resulted in ethylene synthesis, as indicated by the induction of the most important stress induced ethylene biosynthesis genes, and eventually could lead to root growth inhibition. Additionally, in leaves a lack of APX1 stimulated *ZAT7* transcription that is involved in the activation of defense responses, while a loss of APX2 stimulated synthesis of antioxidant metabolites, resulting in less sensitivity to Cd stress in *ko-apx* mutants in this experimental set-up.

Chapter 4

4.1. Introduction

Environmental pollution by metals, such as copper (Cu), has significantly increased during the 19th and 20th century, mainly due to anthropogenic activities such as industrial and agricultural practices. Metals can cause serious problems for all organisms when exceeding the natural emissions. They can accumulate in crops grown on metal-contaminated soils with negative consequences for the quality and safety of feed and food crops. This in turn is dangerous for food contamination and thus human health (Jarüp 2003, Islam *et al.* 2007, Cockell *et al.* 2008).

Copper is an essential micronutrient for normal growth and development of plants, where it functions as a redox-active transition metal in its multiple oxidation states in many physiological processes (Yruela 2005, Yruela 2009). However, the redox properties that make Cu an essential element, also contribute to its inherent toxicity. Excess Cu causes disorders in plant growth and development by adversely affecting important physiological processes (Cuypers *et al.* 2005, Yruela 2009). Redox-active transition metals like Cu catalyze the formation of hydroxyl radicals (°OH) in the non-enzymatic chemical reaction between $O_2°^-$ (superoxide) and H_2O_2 (hydrogen peroxide), called the Fenton and Haber-Weiss reactions (Yruela 2005, Yruela 2009). As such, exposure to Cu can lead to oxidative stress, one of the primary effects generated by excess metals. During oxidative stress, reactive oxygen species (ROS) (e.g. $O_2°^-$, H_2O_2, °OH) are formed and thereby shift the redox balance to the pro-oxidative side, causing oxidative damage (Fargasova 2001, Arora *et al.* 2002, Smeets *et al.* 2009, Cuypers *et al.* 2012b). Because of the redox-active properties of Cu, it is necessary to scavenge free Cu ions present in plant cells. It is known that excessive amounts of metals can be detoxified by chelation via ligands, for example metallothioneins play a primary role in detoxification of excess Cu (Guo *et al.* 2008). Furthermore, plant cells also contain a well-equipped antioxidative defense system to counterbalance the increase in ROS and maintain the cellular redox homeostasis (Sanita di Toppi and Gabbrielli 1999, Hall 2002, Halliwell 2006). This defense system consists of enzymatic components such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) and non-enzymatic components or metabolites, such as glutathione (GSH) and ascorbate (AsA) (Arora *et al.* 2002, Halliwell 2006).

Whereas elevated ROS levels are linked to harmful stress conditions, controlled levels of ROS are also essential for modulating signaling networks that control both physiological processes and stress responses (Mittler *et al.* 2004, Halliwell 2006). A well-known example of ROS involved in the control of such processes and responses is H_2O_2. As a signaling molecule, H_2O_2 can control cell proliferation and cell death to regulate plant growth and development, as well as the adaptation to abiotic stress factors and proper responses to pathogen attack (Quan *et al.* 2008, Miller *et al.* 2010).

Important scavengers of H_2O_2 are APX and CAT, responsible for the reduction of H_2O_2 to water. Ascorbate peroxidases reduce H_2O_2 using AsA as electron donor in the AsA-GSH cycle. Moreover, in the presence of AsA, APX has a higher substrate affinity for H_2O_2 in comparison to CAT. Therefore APX is able to detoxify low concentrations of H_2O_2 and as such is involved in the fine modulation of ROS for signaling (Mittler 2002). Thus, APX is responsible for protection against harmful amounts of H_2O_2 as well as for the regulation of H_2O_2 levels for signaling (Rizhsky *et al.* 2002, Cuypers *et al.* 2011, Caverzan *et al.* 2012). Different isoforms of APX are present in different cell compartments namely the cytosol, mitochondria, chloroplast and peroxisomes (Gest *et al.* 2013). Two APX isoforms located in the cytosol of *Arabidopsis thaliana*, APX1 and APX2, are the isoforms of focus in this study, because both are stress-responsive and have a different abundance. Isoform *APX1* is constitutively expressed in roots, leaves and stems, and its expression is significantly upregulated in response to diverse (a)biotic stress conditions. In contrast, the expression of *APX2* is almost undetected in many plant tissues under normal conditions but is also significantly increased after exposure to (a)biotic stresses (Zimmermann *et al.* 2004, Frank *et al.* 2009).

Cytosolic APX1 is a regulator of the antioxidant defense protecting organelles against oxidative stress under normal and under stress conditions (Davletova *et al.* 2005). Pnueli *et al.* (2003) showed that *APX1* knockout (*ko-apx1*) plants are characterized by an accumulation of H_2O_2 and a decrease in growth and development under normal circumstances, in addition to being more sensitive to light-induced oxidative stress. Cytosolic APX1 was shown to be essential for the protection of thylakoid and stromal/mitochondrial APXs (Davletova *et al.* 2005) as well as the protection of nuclear DNA during light stress (Vanderauwera *et al.*

2011). These results indicate that APX1 activity might be important in cross-compartment protection under oxidative stress generating conditions. Another cytosolic isoform, APX2, is only induced in the response of plants exposed to (a)biotic stress (Rossel *et al.* 2006, Koussevitzky *et al.* 2008). The expression of a cytosolic *APX* in tomato, homologous to *APX2* in *A. thaliana*, was significantly upregulated in pollen during heat stress (Frank *et al*. 2009). In addition, an *APX2*-overexpressor (*A. thaliana* plants) showed enhanced tolerance to drought and high abscisic acid levels (Rossel *et al*. 2006). The plant responses of rice lacking cytosolic *APX2* (*ko-apx2* mutant) versus over-expressing (*apx2-OX*) it were investigated (Zhang *et al.* 2013). The *ko-apx2* mutant plants were more sensitive to salt, drought and cold stress than wild-type plants, in contrast to the *apx2-OX* plants, which were more tolerant to these stresses in comparison with wild-type plants (Zhang *et al.* 2013). However in *A. thaliana* plants, mutants lacking APX2 also had a decreased tolerance to light stress but an increased tolerance to salt and paraquat-induced oxidative stress (Suzuki *et al.* 2013). Thus, a contrasting role for APX2 in the response to these abiotic stresses was observed, which may depend on the plant species studied and the experimental conditions used.

From the above it is clear that APX1 and APX2 are involved in the acclimation of plants to abiotic stress conditions. Furthermore, it was also demonstrated that the expression of *APX1* and *APX2*, in respectively *ko-apx2* and *ko-apx1* mutants, is induced to compensate the loss of the other cytosolic *APX*. Zhang *et al.* (2013) investigated the plant responses of rice lacking cytosolic *APX2*. The expression of *APX1* was markedly induced in *ko-apx2* mutant rice plants compared to the wildtypes during salt, drought and cold stress. In *A. thaliana* plants (ecotype Wassilewskija) there was a more increased transcription of *APX2* in *ko-apx1* mutants compared to wild-type plants during light and heat stress (Asai *et al.* 2004).

It has been demonstrated in multiple studies that APX activity increases during Cu stress in diverse plant species (Gupta *et al.* 1999, Cuypers *et al.* 2000, Tewari *et al.* 2006, Cuypers *et al.* 2011, Thounaojam *et al.* 2012). Furthermore, the transcript levels of both *APX1* and *APX2* were affected in roots and leaves of *A. thaliana* plants after 24 h Cu exposure (Smeets *et al.* 2009; Cuypers *et al.* 2011). Whereas Lee *et al.* (2007a) and Cuypers *et al.* (2011) postulated that

APXs are important H_2O_2 scavengers and hence regulators of signaling pathways under Cu stress, a lot still has to be discovered about the underlying mechanisms of Cu-induced oxidative stress and downstream responses. Therefore, detoxification and signal transduction of Cu-induced oxidative stress was investigated in *APX1* knockout (*ko-apx1*) and *APX2* knockout (*ko-apx2*) *A. thaliana* plants and compared to the responses in wild-type plants, to reveal the potential involvement of APX1 and/or APX2 in Cu-induced responses.

4.2. Experimental design and methodology

To investigate the Cu sensitivity of *A. thaliana* (wildtypes, *ko-apx1* and *ko-apx2*), plants were grown on vertical agar plates to analyze the root growth. After 7 days of growth on control plates, a homogenous subset of the seedlings was transferred to treatment plates covering a concentration range of Cu (0-15 µM $CuSO_4$) and cultured for another 7 days. To unravel a potential role of APX1 and APX2 in Cu-induced oxidative signaling, plants were grown on hydroponics as described previously (Chapter 3, section 3.2). After three weeks the plants were exposed to 2 µM $CuSO_4$ for 2, 24 and 72 h. Besides biomass and metal content, indicators of the cellular redox state were monitored at different biological levels. At transcriptional level, gene expression was analyzed for 1) oxidative stress related genes, 2) genes involved in signal transduction and 3) genes related to ethylene biosynthesis/signaling. At metabolic level, H_2O_2 content, lipid peroxidation and concentration of antioxidative metabolites (AsA and GSH) were investigated.

4.3. Results

4.3.1. Cu accumulation

When 19-days-old *A. thaliana* plants were exposed to 2 µM Cu in hydroponic culture, Cu was almost entirely retained in the roots and just a small part of the Cu ions was transported to the leaves (Figure 4.1A,B). The values found in the non-exposed (0 h) plants were the same as the values of the controls at 2, 24 and 72 h for all genotypes. In the roots, the Cu uptake was already significantly increased after 2 h of exposure, compared to the non-exposed plants, and this was sustained after 24 and 72 h of exposure (Figure 4.1A). A significant increase in Cu accumulation was observed in the leaves of all genotypes exposed to Cu for 24 h and this remained significantly higher in both *ko-apx* mutants after 72 h Cu exposure (Figure 4.1B).

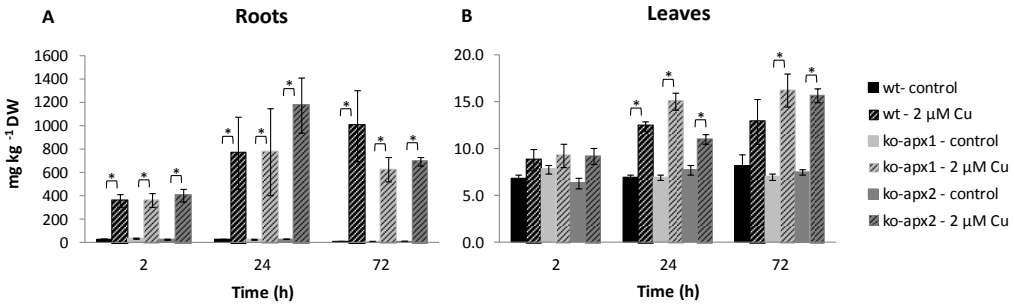

Figure 4.1. Cu accumulation (mg kg^{-1} DW) in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* wild-type, *ko-apx1* and *ko-apx2* plants exposed to 2 µM $CuSO_4$ for 2, 24 and 72 h or grown under control conditions. Values are the mean ± SE of four biological replicates. Significant differences within one time point are shown: *$p<0.05$.

4.3.2. Plant growth

Copper exposure resulted in a growth reduction for both roots and leaves of *A. thaliana* wild-type and mutant plants. A reduction in root fresh weight, but not leaf fresh weight was observed in all genotypes exposed for 24 h to Cu (Figure 4.2A,B). This was concomitant with an increase in the dry weight percentage (%DW) in the roots for all genotypes at this time point (Figure 4.3A). In addition, in leaves of Cu-exposed wildtypes a significant increase in %DW was also observed after 24 h, but not in the knock-out mutants.

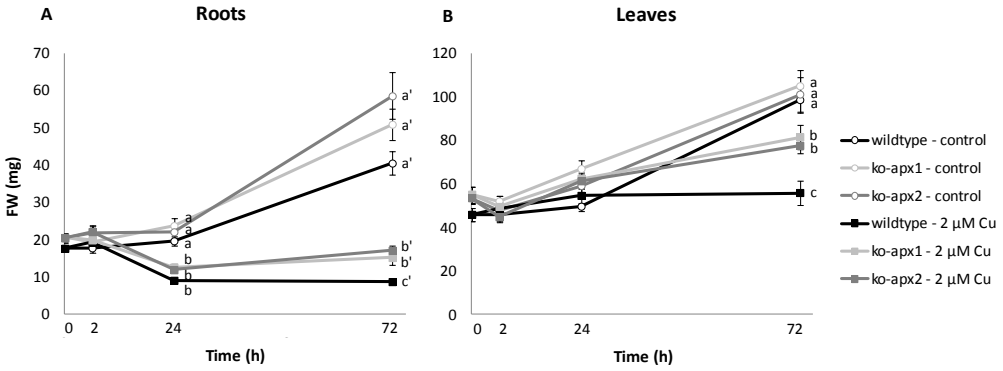

Figure 4.2. Fresh weight (FW, mg) in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* wild-type, *ko-apx1* and *ko-apx2* plants exposed to 2 µM $CuSO_4$ for 0, 2, 24 and 72 h or grown under control conditions. Values are mean ± SE of eight biological replicates. Significant differences within one time point are shown with different letters ($p<0.05$).

All genotypes showed a significantly lower fresh weight of the roots and leaves after 72 h exposure to Cu as compared to the controls (Figure 4.2A,B). Comparing the genotypes after 72 h exposure to Cu, a higher root and leaf fresh weight was observed in *ko-apx1* and *ko-apx2* as compared to wild-type plants (Figure 4.2A,B). After 72 h there was only an increase in root %DW observed in the wildtypes exposed to Cu (Figure 4.3A), whereas %DW in the mutants was restored to normal and no further decline in fresh weight was observed. The leaves of wildtypes exposed to Cu showed a significant increase in %DW after 72 h, which was not observed in the mutants (Figure 4.3B).

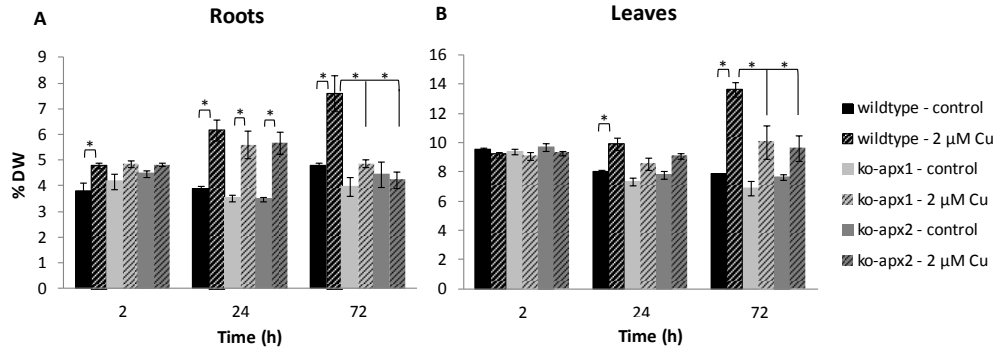

Figure 4.3. Dry weight percentage (%DW) was measured in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* wild-type, *ko-apx1* and *ko-apx2* plants exposed to 2 µM $CuSO_4$ for 2, 24 and 72 h or grown under control conditions. Values are mean ± SE of four biological replicates. Significant differences within one time point are shown: *p<0.05.

Root development during Cu exposure was investigated in more detail in *A. thaliana* wildtypes, *ko-apx1* and *ko-apx2* using a vertical agar plate experimental setup. Plants were germinated on control medium before being transferred 7 days after germination to medium containing increased Cu concentrations. At the time of transfer, primary root length was significantly longer in the *ko-apx1* and *ko-apx2* mutants as compared to the wild-type plants (Figure 4.4A). When plants were exposed to Cu, primary root growth decreased with increasing Cu concentration and no significant differences between the genotypes was observed (Figure 4.4B). The mean length of the lateral roots was significantly higher in the *ko-apx1* mutants compared to the wildtype, which was also found for the *ko-apx2* mutant after exposure to 2 and 10 µM $CuSO_4$ (Figure 4.4C).

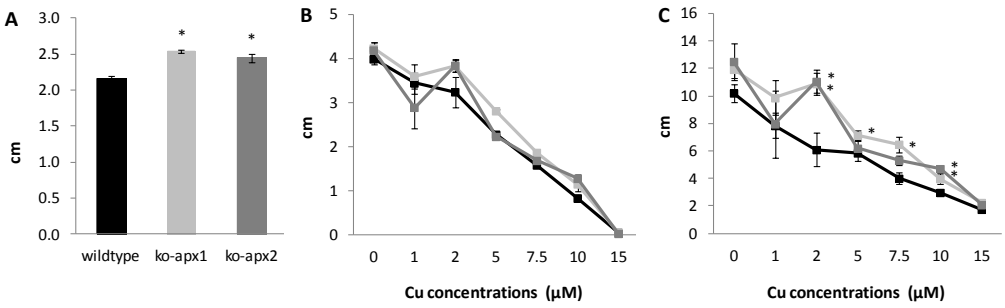

Figure 4.4. Root development parameters in *A. thaliana* wild-type (black), *ko-apx1* (light gray) and *ko-apx2* (dark gray) mutant seedlings after 7 d exposure on vertical agar plates to $CuSO_4$ (0 – 15 µM Cu). Primary root length (cm) before transfer (for the plants transferred to the respective Cu concentrations) (A), primary root length (cm) (B) and average lateral root length (C) after transfer to Cu-containing plates for 7 days. Significant genotype effects within treatments are indicated with respect to the wildtype: *p<0.05.

4.3.3. The cellular redox state of GSH and AsA is affected by Cu exposure

Two major components in the plant antioxidant defense system are the metabolites AsA and glutathione (GSH). The content of both metabolites as well as their redox state were determined in leaves of wild-type, *ko-apx1* and *ko-apx2* plants under normal and Cu-exposed conditions (Table 4.1).

Under normal circumstances, no significant differences in GSH content were observed between the genotypes. When exposed to Cu, wild-type and *ko-apx2* plants showed a treatment effect in the content of total GSH, which was significantly increased in these plants after 72 h. Such a treatment effect was also seen in the levels of reduced GSH, again in wildtypes and *ko-apx2* mutants. Concerning GSSG content, a time effect was noticed in all genotypes with a decrease after 24 h and an increase after 72 h Cu exposure. A similar pattern was also observed for the redox balance in all genotypes.

Under control conditions, no differences in the AsA pool between the genotypes were observed, but under Cu exposure the AsA levels were most strongly affected in *ko-apx2* mutants. Concerning total AsA, a treatment effect (increase) was noticed for wild-type and *ko-apx2* plants. Only for *ko-apx2*, this effect was enhanced in time, and showed a significant increase after 24 and 72 h as compared to their own controls. For the reduced AsA content, an increase after Cu exposure as well as a time effect, was observed in all genotypes. Whereas a significant increase was observed in *ko-apx2* after 24 and 72 h, these effects were less pronounced in wildtypes and *ko-apx1*. A treatment effect in the oxidized form of AsA (DHA) was only observed in the *ko-apx2* mutant. However, increments in both oxidized and reduced ascorbate resulted in a more reduced redox balance in *ko-apx2* mutants during prolonged exposure.

Table 4.1. Metabolite concentrations are expressed as nmoles g^{-1} FW for GSH and as µmoles g^{-1} FW for AsA in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 2 µM $CuSO_4$ for 0, 2, 24 or 72 h. Total metabolite concentrations represent both reduced and oxidized forms; the redox state is the ratio between the oxidized and reduced form. Values are mean ± SE of five biological replicates. Significant differences within one genotype are indicated with highlighted text p<0.05 induction and repression. The letters behind each column represents: A) treatment effect, B) time effect, C) interaction treatment*time.

		wildtype			*ko-apx1*			*ko-apx2*		
		control	2 µM Cu		control	2 µM Cu		control	2 µM Cu	
Total GSH	0h	204.12 ± 27.77			216.69 ± 30.43			218.41 ± 30.35		
	2h	243.09 ± 29.15	251.71 ± 26.06	**A <0.01**	301.80 ± 11.31	227.97 ± 46.21	**A -**	250.42 ± 23.31	291.43 ± 10.02	**A <0.05**
	24h	237.39 ± 8.91	267.06 ± 37.61	**B -**	208.13 ± 21.74	252.39 ± 30.52	**B -**	261.26 ± 22.36	253.75 ± 14.99	**B -**
	72h	197.92 ± 9.66	321.85 ± 37.53	**C -**	211.49 ± 12.21	317.85 ± 37.27	**C <0.05**	180.52 ± 16.57	303.93 ± 48.67	**C -**
GSH	0h	192.18 ± 25.11			205.41 ± 28.44			203.17 ± 28.20		
	2h	230.09 ± 28.86	238.72 ± 25.98	**A <0.01**	284.31 ± 7.31	218.98 ± 44.43	**A -**	238.99 ± 23.95	278.66 ± 10.39	**A <0.01**
	24h	227.17 ± 8.94	264.21 ± 36.84	**B -**	197.41 ± 21.15	247.66 ± 30.34	**B -**	247.05 ± 22.11	250.38 ± 14.96	**B -**
	72h	180.07 ± 11.64	297.76 ± 37.55	**C -**	198.56 ± 11.44	294.39 ± 37.19	**C <0.05**	167.27 ± 16.41	283.66 ± 48.88	**C -**
GSSG	0h	11.94 ± 2.79			14.42 ± 2.53			15.24 ± 3.22		
	2h	13.00 ± 1.45	11.33 ± 1.17	**A -**	12.98 ± 2.22	12.27 ± 2.26	**A -**	11.43 ± 1.30	11.80 ± 0.40	**A -**
	24h	10.22 ± 0.56	2.84 ± 1.37	**B <0.001**	10.11 ± 0.98	4.73 ± 1.16	**B <0.001**	14.20 ± 1.64	3.37 ± 0.53	**B <0.001**
	72h	13.51 ± 2.67	21.11 ± 0.58	**C <0.001**	12.93 ± 1.02	23.46 ± 1.73	**C <0.001**	12.62 ± 0.81	24.50 ± 1.77	**C <0.001**
GSSG/GSH	0h	0.060 ± 0.005			0.057 ± 0.006			0.073 ± 0.010		
	2h	0.061 ± 0.008	0.050 ± 0.010	**A -**	0.059 ± 0.012	0.042 ± 0.005	**A -**	0.054 ± 0.008	0.042 ± 0.002	**A -**
	24h	0.045 ± 0.003	0.017 ± 0.003	**B <0.001**	0.053 ± 0.008	0.021 ± 0.004	**B <0.001**	0.060 ± 0.010	0.014 ± 0.002	**B <0.01**
	72h	0.064 ± 0.003	0.075 ± 0.010	**C -**	0.065 ± 0.004	0.085 ± 0.011	**C <0.05**	0.078 ± 0.009	0.076 ± 0.017	**C -**
Total AsA	0h	2.43 ± 0.48			2.76 ± 0.24			2.92 ± 0.52		
	2h	3.29 ± 0.43	4.39 ± 0.32	**A <0.01**	3.18 ± 0.21	3.76 ± 0.17	**A -**	2.67 ± 0.29	4.19 ± 0.51	**A <0.001**
	24h	3.31 ± 0.78	4.05 ± 0.61	**B -**	4.79 ± 1.45	3.38 ± 0.95	**B -**	1.71 ± 0.11	4.03 ± 0.36	**B <0.05**
	72h	3.88 ± 0.30	5.64 ± 0.85	**C -**	3.77 ± 0.36	5.66 ± 0.66	**C -**	3.12 ± 0.15	5.28 ± 0.67	**C -**
AsA	0h	1.34 ± 0.11			1.48 ± 0.12			1.41 ± 0.13		
	2h	1.59 ± 0.13	1.65 ± 0.24	**A <0.001**	1.52 ± 0.22	1.50 ± 0.16	**A <0.01**	1.62 ± 0.12	1.45 ± 0.19	**A <0.001**
	24h	1.48 ± 0.17	1.87 ± 0.29	**B <0.001**	1.64 ± 0.25	1.87 ± 0.26	**B -**	1.26 ± 0.12	2.24 ± 0.14	**B <0.001**
	72h	1.45 ± 0.18	3.81 ± 0.14	**C <0.001**	1.38 ± 0.16	2.82 ± 0.50	**C <0.05**	1.52 ± 0.13	3.18 ± 0.25	**C <0.001**
DHA	0h	1.08 ± 0.35			1.35 ± 0.15			1.51 ± 0.51		
	2h	1.70 ± 0.49	2.74 ± 0.54	**A -**	1.58 ± 0.31	2.17 ± 0.34	**A -**	1.39 ± 0.18	3.20 ± 0.50	**A <0.05**
	24h	1.83 ± 0.85	2.18 ± 0.85	**B -**	3.15 ± 1.67	1.51 ± 0.89	**B -**	1.43 ± 0.92	1.79 ± 0.46	**B -**
	72h	2.43 ± 0.22	1.83 ± 0.81	**C -**	2.39 ± 0.32	2.84 ± 0.54	**C -**	1.60 ± 0.27	2.10 ± 0.63	**C -**
DHA/AsA	0h	0.78 ± 0.23			1.01 ± 0.19			1.47 ± 0.28		
	2h	1.10 ± 0.35	1.32 ± 0.35	**A -**	1.09 ± 0.33	1.44 ± 0.35	**A -**	0.93 ± 0.21	2.50 ± 0.31	**A -**
	24h	1.33 ± 0.60	1.54 ± 0.81	**B -**	2.26 ± 1.17	1.08 ± 0.58	**B -**	1.20 ± 0.78	0.83 ± 0.23	**B -**
	72h	1.74 ± 0.27	0.48 ± 0.21	**C -**	1.86 ± 0.42	1.20 ± 0.45	**C -**	1.16 ± 0.31	0.67 ± 0.19	**C <0.05**

4.3.4. Visualization of H_2O_2 production by DAB staining

Since H_2O_2 production is a common response to metal exposure, H_2O_2 production was visualized by DAB staining in leaves of wildtypes and *ko-apx* mutants after 2 µM Cu exposure for 2, 24 and 72 h. No visible genotype differences in the appearance of H_2O_2 were found between the leaves of the control groups. When plants were exposed to Cu for 2 h, none of the genotypes presented visible differences between Cu-exposed and non-exposed leaves (data not shown).

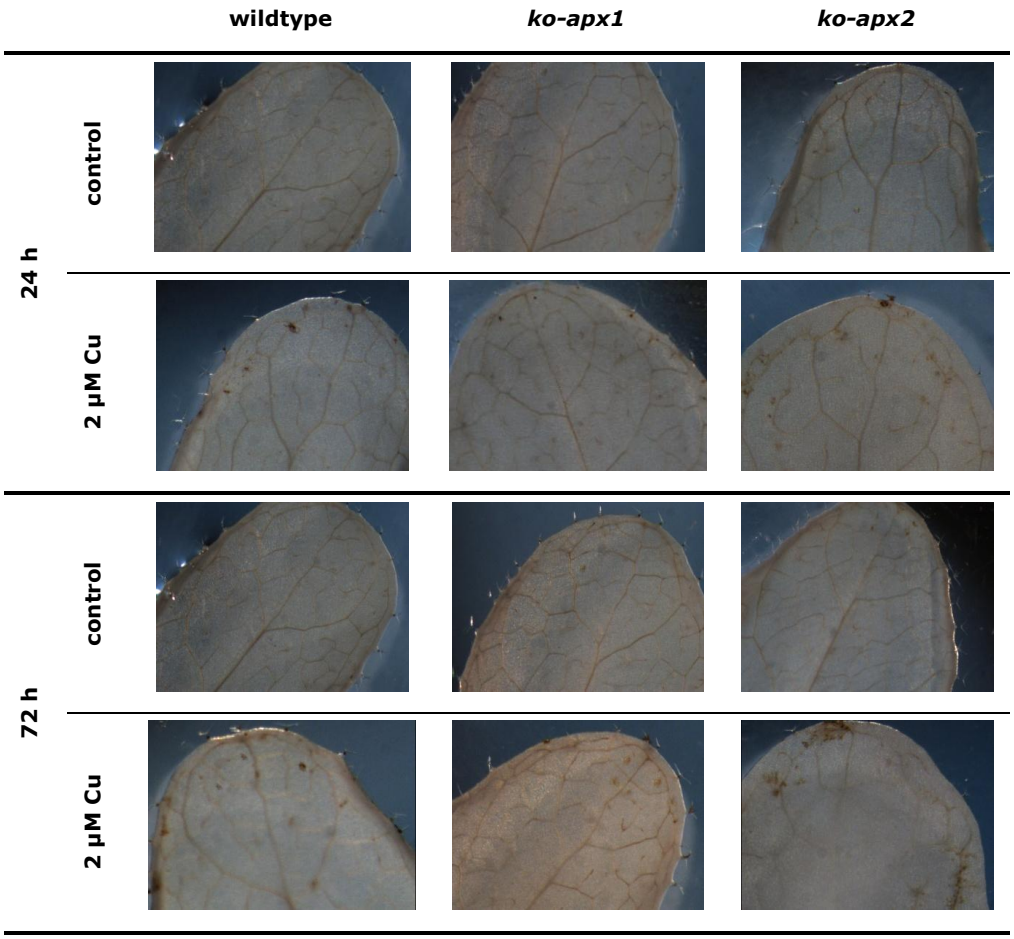

Figure 4.5. Hydrogen peroxide (H_2O_2) staining. The leaves of 19-days-old *Arabidopsis thaliana* genotypes exposed to 2 µM $CuSO_4$ for 2, 24 and 72 h were stained with 3,3'-diaminobenzidine (DAB) to visualize the presence of H_2O_2. Brown spots were found in all genotypes after exposure to Cu, but it was less pronounced in *ko-apx1* leaves.

After 24 h of exposure to Cu, a small increase in H_2O_2 accumulation was observed in the Cu-exposed group compared to the non-exposed group for wild-type plants and *ko-apx2* mutants, while this increase was less pronounced in the leaves of *ko-apx1* (Figure 4.5). This increase in the Cu-exposed group, compared to the non-exposed group, was still observed in the leaves of all genotypes after 72 h, once again less pronounced in the leaves of the *ko-apx1* mutant (Figure 4.5).

4.3.5. Lipid peroxidation in Cu-exposed A. thaliana plants is different from non-exposed plants

Metal-induced membrane damage was measured in roots and leaves based on TBArm levels, which are an indication of lipid peroxidation (Figure 4.6A,B). The values found for the non-exposed (0 h) plants were the same as the values of the controls at 2, 24 and 72 h in all genotypes. In the roots, lipid peroxidation was already significantly elevated after 2 h of Cu exposure in the wild-type plants, but not in the mutants. After 24 h, an elevation in TBArm was observed in all genotypes when exposed to Cu, but the observed lipid peroxidation was significantly lower in the *ko-apx2* roots compared to those of the wildtypes. The measured TBArm in the genotypes exposed to Cu during 72 h were still significantly higher than the unexposed roots, but the TBArm content was the highest after 24 h exposure to Cu.

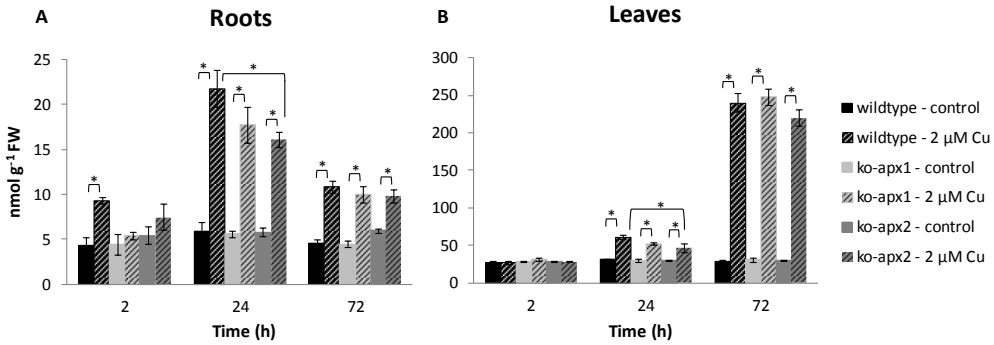

Figure 4.6. The concentrations of TBA reactive metabolites are expressed as nmol g^{-1} FW in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* wild-type, *ko-apx1* and *ko-apx2* plants exposed to 2 µM $CuSO_4$ for 2, 24 and 72 h or grown under control conditions. Values are mean ± SE of four biological replicates. Significant differences within one time point are shown: *p<0.05.

Exposure to Cu increased the lipid peroxidation in the leaves after 24 h in all genotypes, and even more after 72 h exposure. Only after 24 h the TBArm in *ko-apx2* were significantly lower than those observed in the wildtypes.

4.3.6. Alterations in the gene expression network of A. thaliana during Cu stress

4.3.6.1. Oxidative stress markers

Five markers for oxidative stress responses were measured (Gadjev *et al.* 2006), which are to be found in mitochondria (*OX1*), cytoplasm (*OX4*), chloroplast (*OX5*) and extracellular (*OX2*). The latter two are involved in defense responses. The marker *OX3* codes for an unknown protein and its cellular localization is unknown.

In roots (Table 4.2), all oxidative stress markers, except *OX1* transcript levels, were upregulated after 2 h in all genotypes. After 24 h, the expression of all markers, including *OX1*, was still increased in all genotypes with the highest induction for the transcript levels of *OX3* and *OX5*. After 72 h Cu exposure, the expression level of all markers was still induced in the wild-type plants, but the transcript levels of *OX1*, *OX4* and *OX5* were significantly lower in the roots of both *ko-apx* mutants compared to the wildtypes.

In leaves (Table 4.3), the oxidative stress markers were also upregulated after exposure to Cu. The transcript levels of *OX3* and *OX5* were increased already after 2 h in the leaves of the wildtypes and *ko-apx1*, while there was no significant induction in expression in *ko-apx2* leaves. After 24 h there was also an induction in the gene expression of *OX4* in wild-type and *ko-apx1* plants, while once again no induction was observed in *ko-apx2* mutants. Furthermore, the transcript levels of *OX4* and *OX5* were still induced after 72 h in *ko-apx1* and the latter in *ko-apx2*, while no upregulations in these genes were found in the wildtypes at this time point.

Table 4.2. Transcript levels in roots of 19-days-old *Arabidopsis thaliana* plants exposed to 2 µM $CuSO_4$ for 0, 2, 24 or 72 h. Fold changes in expression levels within a genotype and time point are relative to the non-exposed plants (0 µM) (1.00 ± SE). A significant effect of treatment (relatively to 0 h) is indicated with highlighted text: p<0.05 induction, repression; p<0.01 induction, repression; p<0.001 induction, repression. Significant interaction effects (genotype*treatment) within a time point are represented by different lowercase letters. Values are mean ± SE of four biological replicates.

ROOT		wildtype		*ko-apx1*		*ko-apx2*		ROOT		wildtype		*ko-apx1*		*ko-apx2*	
Oxidative stress markers															
OX1	0h	1.00 ± 0.17		1.00 ± 0.06		1.00 ± 0.13		*OX4*	0h	1.00 ± 0.21		1.00 ± 0.14		1.00 ± 0.21	
	2h	2.22 ± 0.18		2.51 ± 0.11		3.02 ± 0.33			2h	17.63 ± 2.97		12.43 ± 1.29		15.88 ± 2.39	
	24h	19.84 ± 1.15		20.88 ± 2.16		24.32 ± 2.12			24h	32.39 ± 3.67		44.15 ± 1.61		41.01 ± 2.13	
	72h	31.66 ± 4.49	a	15.33 ± 8.14	ab	10.35 ± 5.42	b		72h	53.47 ± 7.99	a	16.18 ± 7.66	b	12.27 ± 6.56	b
OX2	0h	1.00 ± 0.05		1.00 ± 0.31		1.00 ± 0.26		*OX5*	0h	1.00 ± 0.22		1.00 ± 0.19		1.00 ± 0.21	
	2h	90.29 ± 23.63		83.75 ± 14.87		115.34 ± 27.33			2h	269.85 ± 14.36		203.50 ± 6.76		332.07 ± 14.86	
	24h	6153.20 ± 747.80		3037.80 ± 116.12		5751.63 ± 189.92			24h	555.48 ± 52.49		345.69 ± 5.55		538.04 ± 66.20	
	72h	7527.39 ± 694.14		7541.61 ± 4011.58		4607.26 ± 2613.86			72h	457.92 ± 80.20	a	89.69 ± 50.75	b	140.75 ± 76.89	b
OX3	0h	1.00 ± 0.05		1.00 ± 0.10		1.00 ± 0.13									
	2h	193.20 ± 9.26		259.50 ± 25.32		236.24 ± 14.74									
	24h	146.23 ± 13.43		167.29 ± 4.57		110.98 ± 12.82									
	72h	78.91 ± 2.73		46.45 ± 26.60		36.70 ± 19.73									
Genes coding for pro-oxidative enzymes															
RBHOD	0h	1.00 ± 0.19		1.00 ± 0.04		1.00 ± 0.03		*LOX1*	0h	1.00 ± 0.08		1.00 ± 0.02		1.00 ± 0.05	
	2h	12.36 ± 0.99		14.15 ± 2.09		19.86 ± 0.95			2h	7.52 ± 0.07		9.59 ± 0.91		12.06 ± 1.23	
	24h	22.85 ± 1.40		20.04 ± 0.30		19.68 ± 1.91			24h	178.97 ± 23.73		208.51 ± 21.30		192.18 ± 17.73	
	72h	8.91 ± 1.33		5.57 ± 2.44		6.31 ± 2.93			72h	47.39 ± 5.34		27.39 ± 11.27		25.78 ± 13.85	
Genes coding for AsA or GSH biosynthesis enzymes															
GME	0h	1.00 ± 0.03		1.00 ± 0.02		1.00 ± 0.06		*GSH1*	0h	1.00 ± 0.08		1.00 ± 0.04		1.00 ± 0.05	
	2h	1.02 ± 0.05		0.97 ± 0.02		1.00 ± 0.04			2h	2.17 ± 0.17		2.18 ± 0.19		2.43 ± 0.17	
	24h	0.86 ± 0.05		0.87 ± 0.04		0.93 ± 0.05			24h	1.80 ± 0.29		1.76 ± 0.06		1.86 ± 0.16	
	72h	1.12 ± 0.11	a	0.99 ± 0.09	ab	0.76 ± 0.03	b		72h	1.51 ± 0.25		0.97 ± 0.10		0.99 ± 0.12	
VTC1	0h	1.00 ± 0.04		1.00 ± 0.01		1.00 ± 0.05		*GSH2*	0h	1.00 ± 0.07		1.00 ± 0.02		1.00 ± 0.03	
	2h	2.19 ± 0.07		2.22 ± 0.18		3.05 ± 0.13			2h	3.30 ± 0.12		3.55 ± 0.19		4.34 ± 0.29	
	24h	2.70 ± 0.34		3.37 ± 0.18		2.81 ± 0.17			24h	4.13 ± 0.54		3.53 ± 0.14		3.80 ± 0.29	
	72h	2.38 ± 0.26	a	1.43 ± 0.19	b	1.34 ± 0.29	b		72h	2.02 ± 0.24	a	1.20 ± 0.17	b	1.15 ± 0.21	b
Genes coding for antioxidant enzymes															
CSD1	0h	1.00 ± 0.04		1.00 ± 0.05		1.00 ± 0.05		*FSD1*	0h	1.00 ± 0.08		1.00 ± 0.08		1.00 ± 0.02	
	2h	1.57 ± 0.06		1.64 ± 0.11		1.77 ± 0.09			2h	0.99 ± 0.15	ab	1.43 ± 0.07	a	0.81 ± 0.14	b
	24h	3.36 ± 0.18		3.07 ± 0.30		3.22 ± 0.04			24h	0.06 ± 0.01		0.04 ± 0.02		0.03 ± 0.01	
	72h	2.27 ± 0.18		1.94 ± 0.35		1.95 ± 0.37			72h	0.01 ± 0.00		0.01 ± 0.00		0.00 ± 0.00	
CSD2	0h	1.00 ± 0.03		1.00 ± 0.07		1.00 ± 0.06		*CAT1*	0h	1.00 ± 0.07		1.00 ± 0.03		1.00 ± 0.04	
	2h	1.24 ± 0.06		0.94 ± 0.02		1.39 ± 0.15			2h	2.23 ± 0.28		2.70 ± 0.49		2.40 ± 0.18	
	24h	0.95 ± 0.02		0.87 ± 0.14		0.91 ± 0.03			24h	22.32 ± 2.83		19.00 ± 3.07		24.10 ± 1.44	
	72h	1.06 ± 0.10		1.36 ± 0.04		1.52 ± 0.12			72h	15.70 ± 1.47		10.24 ± 4.45		7.95 ± 3.91	

ROOT		wildtype		ko-apx1		ko-apx2		ROOT		wildtype		ko-apx1		ko-apx2	
Genes coding for antioxidant enzymes															
CAT2	0h	1.00 ± 0.08		1.00 ± 0.14		1.00 ± 0.06		*GR1*	0h	1.00 ± 0.01		1.00 ± 0.04		1.00 ± 0.05	
	2h	0.69 ± 0.08		0.61 ± 0.05		0.54 ± 0.09			2h	1.20 ± 0.06		1.51 ± 0.08		1.42 ± 0.12	
	24h	0.32 ± 0.03		0.21 ± 0.02		0.36 ± 0.05			24h	3.18 ± 0.40		2.97 ± 0.38		3.50 ± 0.66	
	72h	0.22 ± 0.01		0.39 ± 0.10		0.34 ± 0.09			72h	2.22 ± 0.18		1.49 ± 0.32		1.60 ± 0.40	
CAT3	0h	1.00 ± 0.10		1.00 ± 0.09		1.00 ± 0.02		*GR2*	0h	1.00 ± 0.06		1.00 ± 0.04		1.00 ± 0.04	
	2h	0.96 ± 0.07		0.95 ± 0.08		0.91 ± 0.05			2h	0.72 ± 0.04		0.81 ± 0.03		0.73 ± 0.06	
	24h	0.68 ± 0.13	a	0.29 ± 0.01	b	0.57 ± 0.14	a		24h	0.69 ± 0.04		0.57 ± 0.01		0.73 ± 0.01	
	72h	11.87 ± 0.87		6.94 ± 2.81		8.09 ± 3.95			72h	0.94 ± 0.07		0.71 ± 0.06		0.71 ± 0.08	
APX1	0h	1.00 ± 0.01				1.00 ± 0.08		*DHAR1*	0h	1.00 ± 0.04		1.00 ± 0.02		1.00 ± 0.05	
	2h	1.56 ± 0.07				1.77 ± 0.14			2h	1.02 ± 0.05		0.98 ± 0.06		1.05 ± 0.07	
	24h	2.00 ± 0.17				2.16 ± 0.20			24h	0.53 ± 0.02		0.54 ± 0.03		0.59 ± 0.03	
	72h	1.22 ± 0.08				0.90 ± 0.06			72h	1.25 ± 0.14		1.06 ± 0.07		0.97 ± 0.08	
APX2	0h	1.00 ± 0.16		1.00 ± 0.15											
	2h	0.83 ± 0.13	a	2.32 ± 0.06	b										
	24h	3.29 ± 0.82		3.83 ± 1.09											
	72h	4.72 ± 1.08		1.61 ± 0.69											
Genes coding for regulatory enzymes															
OXI1	0h	1.00 ± 0.04		1.00 ± 0.11		1.00 ± 0.09		*ZAT7*	0h	1.00 ± 0.10		1.00 ± 0.03		1.00 ± 0.40	
	2h	63.39 ± 2.88		52.89 ± 8.72		114.10 ± 4.04			2h	556.38 ± 25.10		698.60 ± 108.58		585.16 ± 59.88	
	24h	56.57 ± 6.86		79.42 ± 1.30		79.18 ± 9.05			24h	812.54 ± 133.44		709.83 ± 32.11		498.23 ± 54.16	
	72h	25.04 ± 4.36		16.06 ± 8.77		14.55 ± 7.60			72h	253.79 ± 48.58		206.70 ± 122.50		131.43 ± 73.32	
MPK3	0h	1.00 ± 0.09		1.00 ± 0.07		1.00 ± 0.06		*ZAT10*	0h	1.00 ± 0.19		1.00 ± 0.08		1.00 ± 0.19	
	2h	11.21 ± 0.33		11.98 ± 1.36		11.73 ± 1.31			2h	46.63 ± 2.19		100.68 ± 21.64		90.48 ± 7.85	
	24h	3.93 ± 0.28		4.51 ± 0.39		3.79 ± 0.49			24h	38.57 ± 2.87		32.93 ± 0.27		29.19 ± 2.54	
	72h	3.45 ± 0.33		1.83 ± 0.36		1.76 ± 0.47			72h	39.49 ± 4.01	a	15.95 ± 8.13	ab	14.02 ± 7.11	b
MPK4	0h	1.00 ± 0.01		1.00 ± 0.02		1.00 ± 0.04		*ZAT12*	0h	1.00 ± 0.02		1.00 ± 0.05		1.00 ± 0.07	
	2h	1.45 ± 0.00		1.50 ± 0.01		1.54 ± 0.04			2h	111.83 ± 4.83		98.56 ± 3.23		115.38 ± 8.10	
	24h	1.18 ± 0.07		1.09 ± 0.06		1.03 ± 0.09			24h	126.29 ± 9.98		123.08 ± 6.46		121.50 ± 16.06	
	72h	1.28 ± 0.06	a	0.96 ± 0.01	b	0.92 ± 0.05	b		72h	61.68 ± 8.68		40.32 ± 22.06		33.05 ± 18.02	
MPK6	0h	1.00 ± 0.02		1.00 ± 0.03		1.00 ± 0.05		*HSF21*	0h	1.00 ± 0.06		1.00 ± 0.08		1.00 ± 0.10	
	2h	1.15 ± 0.07		1.20 ± 0.02		1.26 ± 0.07			2h	7.04 ± 0.32		7.49 ± 0.17		9.73 ± 0.55	
	24h	1.56 ± 0.15		1.38 ± 0.02		1.58 ± 0.08			24h	8.23 ± 1.23		9.27 ± 0.20		10.45 ± 1.21	
	72h	1.25 ± 0.13		1.03 ± 0.08		1.16 ± 0.00			72h	4.98 ± 0.83		2.70 ± 0.93		2.82 ± 0.96	
WRKY22	0h	1.00 ± 0.11		1.00 ± 0.10		1.00 ± 0.08									
	2h	3.54 ± 0.00		4.69 ± 0.44		5.83 ± 0.63									
	24h	8.86 ± 1.42		10.10 ± 0.92		8.41 ± 1.36									
	72h	3.23 ± 0.08		1.71 ± 0.72		1.73 ± 0.78									
Genes involved in the ethylene biosynthesis/signaling															
ACS2	0h	1.00 ± 0.07		1.00 ± 0.11		1.00 ± 0.08		*ERF1*	0h	1.00 ± 0.12		1.00 ± 0.09		1.00 ± 0.06	
	2h	4.18 ± 0.61		7.85 ± 1.44		11.75 ± 1.98			2h	115.17 ± 5.59		150.28 ± 34.91		192.51 ± 13.16	
	24h	290.27 ± 28.56		332.68 ± 25.64		307.31 ± 45.99			24h	202.72 ± 7.51		82.63 ± 24.02		137.71 ± 10.14	
	72h	111.07 ± 10.43		49.05 ± 28.80		41.79 ± 23.73			72h	90.02 ± 10.60	a	53.43 ± 31.41	ab	27.04 ± 14.89	b
ACS6	0h	1.00 ± 0.06		1.00 ± 0.06		1.00 ± 0.04									
	2h	26.54 ± 1.49		42.04 ± 2.25		49.72 ± 4.15									
	24h	22.15 ± 2.32		22.85 ± 0.61		24.19 ± 1.79									
	72h	9.87 ± 1.91	a	4.71 ± 2.06	ab	3.63 ± 1.48	b								

4.3.6.2. Pro-oxidative genes

To investigate the involvement of pro-oxidative genes in Cu-induced ROS production, Remans *et al.* (2010) measured the expression of different members of lipoxygenases (LOXs) and NADPH oxidases in *A. thaliana*, which were influenced during Cu stress. In this study, the expression of the cytosolic isoform *LOX1* and plastidic isoform *LOX2* was analyzed. The gene expression of *RBOHD* (respiratory burst oxidase homologue D), a member of the NADPH oxidase gene family, was measured as well, because a link between *RBOHD* and *APX* was observed by Davletova *et al.* (2005) and Bechtold *et al.* (2008).

In roots (Table 4.2), the mRNA level of *LOX1* was increased for all genotypes at all time points, peaking after 24 h. Also, the expression of *RBOHD* was significantly increased after 2 h Cu exposure in all genotypes and persisted and peaked after 24 h. Only in the wildtypes *RBOHD* transcript levels were still significantly enhanced after 72 h.

In leaves (Table 4.3), there was a transient induction of *LOX2* in all genotypes, whereas no significant differences were observed for *LOX1*. Also a significant increase in *RBOHD* was observed in all genotypes after 2 h, but the rise in Cu-exposed *ko-apx2* leaves was significantly lower than those of the wildtypes.

4.3.6.3. Antioxidative gene expression

A number of antioxidative genes were measured in roots and leaves of all genotypes. These included different isoforms of SOD and CAT as well as the cytosolic isoforms of APX. Furthermore, the genes involved in the biosynthesis of AsA [*i.e.* GDP-mannose epimerase (*GME*) and GDP-mannose pyrophosphorylase (*VTC1*)] and GSH [*i.e.* gamma-glutamylcysteine synthetase (*GSH1*) and glutathione synthetase (*GSH2*)], as well as genes involved in the AsA-GSH cycle [*i.e.* dehydroascorbate dehydrogenase (*DHAR*) and glutathione reductase (*GR*)] were analyzed.

Under control conditions, the expression of *CAT2* was constitutively higher in *ko-apx1* roots as compared to the other two genotypes (Table 4.4). In the roots, genes involved in AsA (*VTC1*) and GSH (*GSH1,GSH2*) biosynthesis responded fast (2 h) to Cu exposure and the increased expression was also sustained at 24 h Cu exposure (Table 4.2). In the wildtypes, an induction in the expression of *VTC1* and *GSH2* remained at 72 h, in contrast to both *ko-apx* mutants that had a

significantly lower expression under Cu exposure. Looking at the gene expression level of SODs, an increase in *CSD1* was observed after 2 h Cu exposure in all genotypes, at least significant for the mutants, and was sustained after 24 and 72 h in all genotypes. In contrast, a decrease was seen for *FSD1* in all genotypes after 24 h and 72 h Cu exposure. Also, a different expression was shown in the *CAT* family. In all genotypes, transcripts of *CAT1* were increased, while *CAT2* showed a decreased transcript level after 24 and 72 h Cu exposure. The expression of *APX2* was increased in *ko-apx1* after exposure to Cu for 2 h and was still induced after 24 h. In the wildtypes, an increased *APX2* transcript level was observed after 24 h and still sustained after 72 h. The transcript levels of *CAT1*, *CSD1*, *APX1*, *GR1* and *GSH2* peaked after 24 h exposure to Cu in all genotypes. Some antioxidative genes were only induced in the wildtypes after 72 h, *i.e. CAT3*, *APX2* and *GR1*.

In contrast to the roots, the leaves showed no major significant differences in the expression of antioxidative genes when exposed to Cu. Similar to the roots, leaves of all genotypes showed an increase in *CSD1*, while a decrease was observed in *FSD1* after Cu exposure. Opposite expression levels were also seen for *CAT2* (increase) and *CAT3* (decrease) after Cu exposure.

4.3.6.4. Expression of genes involved in oxidative signaling

Hydrogen peroxide can be harmful for the plant but it can also act as a signaling molecule in mediating defense responses against abiotic stresses. The expression levels of genes involved in H_2O_2 signaling that were previously found responsive to Cu exposure in *A. thaliana* (Opdenakker *et al.* 2012a) were determined in *ko-apx* genotypes and compared to wildtypes (Table 4.2, 4.3). In general, induced gene expression levels were observed in both roots and leaves already after 2 h exposure to Cu. Since it is known that H_2O_2 is involved in the activation of serine/threonine kinase OXI1 (oxidative signal-inducible 1), we measured *OXI1* gene expression, as well as the expression of genes downstream in the OXI1 signaling cascade.

Table 4.3. Transcript levels in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 2 µM $CuSO_4$ for 0, 2, 24 or 72 h. Fold changes in expression levels within a genotype and time point are relative to the non-exposed plants (0 µM) (1.00 ± SE). A significant effect of treatment (relatively to 0 h) is indicated with highlighted text: $p<0.05$ induction, repression; $p<0.01$ induction, repression; $p<0.001$ induction, repression. Significant interaction effects (genotype*treatment) within a time point are represented by different lowercase letters. Values are mean ± SE of four biological replicates.

LEAF		wildtype		ko-apx1		ko-apx2		LEAF		wildtype		ko-apx1		ko-apx2	
Oxidative stress markers															
OX1	0h	1.00 ± 0.10		1.00 ± 0.03		1.00 ± 0.10		*OX4*	0h	1.00 ± 0.22		1.00 ± 0.22		1.00 ± 0.08	
	2h	1.04 ± 0.06		1.10 ± 0.07		1.16 ± 0.11			2h	1.45 ± 0.17		1.66 ± 0.40		1.48 ± 0.08	
	24h	0.66 ± 0.07		0.69 ± 0.07		0.92 ± 0.09			24h	3.56 ± 0.73		3.82 ± 0.71		1.62 ± 0.22	
	72h	0.79 ± 0.05		0.84 ± 0.08		0.92 ± 0.09			72h	1.43 ± 0.13		3.09 ± 0.30		1.70 ± 0.29	
OX2	0h	1.00 ± 0.26		1.00 ± 0.17		1.00 ± 0.09		*OX5*	0h	1.00 ± 0.48		1.00 ± 0.45		1.00 ± 0.43	
	2h	2.43 ± 0.56		2.73 ± 0.78		1.47 ± 0.01			2h	12.76 ± 3.04	a	27.52 ± 10.47	a	0.83 ± 0.35	b
	24h	13.35 ± 1.64		6.40 ± 1.17		5.63 ± 0.79			24h	10.35 ± 1.01		12.04 ± 2.20		7.66 ± 2.35	
	72h	3.04 ± 0.48		5.05 ± 0.66		3.89 ± 1.05			72h	3.85 ± 0.88		15.57 ± 1.50		7.56 ± 1.69	
OX3	0h	1.00 ± 0.31		1.00 ± 0.31		1.00 ± 0.02									
	2h	33.46 ± 5.46		38.28 ± 1.83		12.45 ± 3.84									
	24h	3.86 ± 0.04		4.58 ± 0.60		2.96 ± 0.47									
	72h	3.71 ± 0.90		4.89 ± 0.30		3.93 ± 0.64									
Genes coding for pro-oxidant enzymes															
RBHOD	0h	1.00 ± 0.11		1.00 ± 0.04		1.00 ± 0.04		*LOX2*	0h	1.00 ± 0.23		1.00 ± 0.20		1.00 ± 0.09	
	2h	4.01 ± 0.57	a	3.14 ± 0.46	ab	1.93 ± 0.30	b		2h	1.85 ± 0.15		1.64 ± 0.48		1.25 ± 0.33	
	24h	2.09 ± 0.15		2.03 ± 0.26		2.45 ± 0.42			24h	5.43 ± 0.78		3.87 ± 0.79		3.63 ± 0.48	
	72h	1.98 ± 0.24		1.92 ± 0.41		1.86 ± 0.36			72h	2.93 ± 0.56		2.83 ± 0.89		2.51 ± 1.04	
LOX1	0h	1.00 ± 0.06		1.00 ± 0.08		1.00 ± 0.09									
	2h	1.21 ± 0.06		1.31 ± 0.00		1.24 ± 0.18									
	24h	1.03 ± 0.14		1.03 ± 0.06		1.10 ± 0.03									
	72h	0.96 ± 0.12		1.33 ± 0.04		1.26 ± 0.07									
Genes coding for AsA or GSH biosynthesis enzymes															
GME	0h	1.00 ± 0.11		1.00 ± 0.01		1.00 ± 0.02		*GSH1*	0h	1.00 ± 0.17		1.00 ± 0.06		1.00 ± 0.09	
	2h	0.91 ± 0.03		0.90 ± 0.03		1.01 ± 0.04			2h	1.33 ± 0.04		1.18 ± 0.05		1.18 ± 0.07	
	24h	0.58 ± 0.06	a	0.78 ± 0.03	ab	0.91 ± 0.08	b		24h	0.98 ± 0.13		1.19 ± 0.04		1.34 ± 0.10	
	72h	0.65 ± 0.02		0.76 ± 0.08		0.64 ± 0.07			72h	0.83 ± 0.11		0.97 ± 0.05		0.82 ± 0.09	
VTC1	0h	1.00 ± 0.10		1.00 ± 0.05		1.00 ± 0.04		*GSH2*	0h	1.00 ± 0.12		1.00 ± 0.03		1.00 ± 0.10	
	2h	1.44 ± 0.14		1.56 ± 0.33		1.06 ± 0.01			2h	1.53 ± 0.08		1.59 ± 0.13		1.29 ± 0.17	
	24h	0.94 ± 0.09		1.30 ± 0.07		1.14 ± 0.08			24h	1.17 ± 0.16		1.11 ± 0.02		1.25 ± 0.14	
	72h	1.28 ± 0.14		1.13 ± 0.17		1.02 ± 0.08			72h	1.03 ± 0.17		1.20 ± 0.08		1.04 ± 0.03	
Genes coding for antioxidant enzymes															
CSD1	0h	1.00 ± 0.13		1.00 ± 0.06		1.00 ± 0.06		*CSD2*	0h	1.00 ± 0.08		1.00 ± 0.07		1.00 ± 0.18	
	2h	1.05 ± 0.17		0.79 ± 0.01		0.93 ± 0.19			2h	0.87 ± 0.15		0.64 ± 0.07		1.03 ± 0.38	
	24h	1.72 ± 0.01		1.81 ± 0.18		1.67 ± 0.10			24h	0.59 ± 0.05		0.91 ± 0.09		0.90 ± 0.17	
	72h	1.80 ± 0.22		1.55 ± 0.05		2.05 ± 0.06			72h	0.93 ± 0.15		0.75 ± 0.09		1.23 ± 0.35	

LEAF		wildtype		ko-apx1		ko-apx2		LEAF		wildtype		ko-apx1		ko-apx2	
Genes coding for antioxidant enzymes															
FSD1	0h	1.00 ± 0.01		1.00 ± 0.12		1.00 ± 0.06		*APX1*	0h	1.00 ± 0.18				1.00 ± 0.07	
	2h	0.58 ± 0.26		0.92 ± 0.22		1.05 ± 0.13			2h	1.08 ± 0.11				1.13 ± 0.12	
	24h	0.07 ± 0.00		0.07 ± 0.01		0.10 ± 0.02			24h	1.20 ± 0.09				1.48 ± 0.09	
	72h	0.03 ± 0.01		0.01 ± 0.00		0.02 ± 0.01			72h	1.18 ± 0.19				0.91 ± 0.12	
CAT1	0h	1.00 ± 0.10		1.00 ± 0.04		1.00 ± 0.09		*APX2*	0h	1.00 ± 0.13		1.00 ± 0.16			
	2h	1.38 ± 0.18		1.58 ± 0.09		1.37 ± 0.10			2h	0.87 ± 0.24		0.77 ± 0.18			
	24h	1.12 ± 0.05		0.98 ± 0.05		1.30 ± 0.09			24h	2.75 ± 0.29		2.24 ± 0.44			
	72h	0.72 ± 0.14	a	1.21 ± 0.16	b	1.08 ± 0.07	ab		72h	0.65 ± 0.23		0.88 ± 0.28			
CAT2	0h	1.00 ± 0.08		1.00 ± 0.07		1.00 ± 0.10		*GR1*	0h	1.00 ± 0.02		1.00 ± 0.08		1.00 ± 0.06	
	2h	0.81 ± 0.04		0.82 ± 0.06		0.74 ± 0.06			2h	1.20 ± 0.02		1.18 ± 0.07		0.93 ± 0.07	
	24h	0.64 ± 0.02		0.75 ± 0.06		0.85 ± 0.08			24h	1.13 ± 0.10		1.20 ± 0.06		1.30 ± 0.05	
	72h	0.39 ± 0.02		0.46 ± 0.06		0.46 ± 0.08			72h	0.66 ± 0.09	a	0.93 ± 0.06	b	0.81 ± 0.06	ab
CAT3	0h	1.00 ± 0.09		1.00 ± 0.04		1.00 ± 0.05		*GR2*	0h	1.00 ± 0.17		1.00 ± 0.09		1.00 ± 0.08	
	2h	1.38 ± 0.07		1.60 ± 0.11		1.08 ± 0.16			2h	1.25 ± 0.06		1.50 ± 0.07		1.20 ± 0.15	
	24h	3.93 ± 0.27	a	1.64 ± 0.46	b	3.09 ± 0.52	ab		24h	0.86 ± 0.08		0.90 ± 0.06		0.91 ± 0.05	
	72h	2.82 ± 0.44		3.20 ± 0.76		2.33 ± 0.71			72h	0.56 ± 0.12		0.70 ± 0.06		0.79 ± 0.06	
DHAR1	0h	1.00 ± 0.20		1.00 ± 0.05		1.00 ± 0.12									
	2h	0.76 ± 0.03		0.62 ± 0.05		0.93 ± 0.20									
	24h	0.49 ± 0.06		0.87 ± 0.10		0.87 ± 0.18									
	72h	0.89 ± 0.13		0.59 ± 0.05		0.64 ± 0.04									
Genes coding for regulatory enzymes															
OXI1	0h	1.00 ± 0.26		1.00 ± 0.03		1.00 ± 0.32		*ZAT7*	0h	1.00 ± 0.35		1.00 ± 0.34		1.00 ± 0.08	
	2h	39.30 ± 12.19	a	23.68 ± 3.08	a	5.02 ± 2.44	b		2h	14.13 ± 3.38	a	27.47 ± 4.82	a	2.17 ± 0.75	b
	24h	4.78 ± 0.51		2.15 ± 0.04		3.34 ± 0.47			24h	3.87 ± 0.09		5.47 ± 1.17		4.48 ± 1.32	
	72h	1.18 ± 0.24		1.85 ± 0.20		4.38 ± 1.59			72h	0.66 ± 0.23	a'	9.47 ± 3.07	b'	3.05 ± 0.66	ab'
MPK3	0h	1.00 ± 0.22		1.00 ± 0.12		1.00 ± 0.09		*ZAT10*	0h	1.00 ± 0.30		1.00 ± 0.04		1.00 ± 0.12	
	2h	7.18 ± 1.09	a	7.85 ± 0.50	a	2.07 ± 0.34	b		2h	10.89 ± 2.46		31.97 ± 1.44		9.94 ± 0.91	
	24h	2.82 ± 0.20		2.75 ± 0.10		3.66 ± 0.44			24h	4.92 ± 0.50		4.16 ± 0.80		3.56 ± 0.57	
	72h	1.81 ± 0.44		1.96 ± 0.21		2.91 ± 0.58			72h	7.79 ± 3.09		3.47 ± 1.01		4.97 ± 0.97	
MPK4	0h	1.00 ± 0.17		1.00 ± 0.06		1.00 ± 0.07		*ZAT12*	0h	1.00 ± 0.29		1.00 ± 0.20		1.00 ± 0.10	
	2h	1.30 ± 0.03		1.15 ± 0.06		1.06 ± 0.06			2h	4.20 ± 1.03		6.62 ± 1.48		2.75 ± 0.75	
	24h	1.04 ± 0.03		1.34 ± 0.11		1.36 ± 0.07			24h	3.91 ± 0.55		1.65 ± 0.15		1.69 ± 0.25	
	72h	0.83 ± 0.06		0.95 ± 0.08		1.06 ± 0.06			72h	2.96 ± 1.35		1.38 ± 0.09		1.63 ± 0.07	
MPK6	0h	1.00 ± 0.10		1.00 ± 0.03		1.00 ± 0.07		*HSF21*	0h	1.00 ± 0.20		1.00 ± 0.18		1.00 ± 0.14	
	2h	1.30 ± 0.05		1.27 ± 0.03		1.14 ± 0.06			2h	5.67 ± 0.56	a	3.10 ± 0.63	ab	1.90 ± 0.25	b
	24h	0.92 ± 0.07		1.05 ± 0.07		1.01 ± 0.07			24h	2.86 ± 0.26		3.89 ± 0.29		3.27 ± 0.37	
	72h	0.85 ± 0.12		1.05 ± 0.07		1.04 ± 0.03			72h	2.44 ± 0.58		2.47 ± 0.17		3.67 ± 0.89	
WRKY22	0h	1.00 ± 0.10		1.00 ± 0.06		1.00 ± 0.05									
	2h	6.52 ± 1.11	a	8.34 ± 0.29	a	2.40 ± 0.71	b								
	24h	0.45 ± 0.04		0.59 ± 0.01		0.84 ± 0.10									
	72h	0.63 ± 0.14		0.85 ± 0.04		0.80 ± 0.07									
Genes involved in the ethylene biosynthesis/signaling															
ACS2	0h	1.00 ± 0.38		1.00 ± 0.60		1.00 ± 0.01		*ERF1*	0h	1.00 ± 0.32		1.00 ± 0.36		1.00 ± 0.28	
	2h	3.94 ± 0.58	a	8.45 ± 0.81	a	0.99 ± 0.23	b		2h	16.98 ± 3.86		5.52 ± 1.46		2.65 ± 0.94	
	24h	11.63 ± 2.45		7.60 ± 0.82		6.74 ± 1.64			24h	47.72 ± 6.60		13.17 ± 4.15		20.02 ± 5.05	
	72h	1.47 ± 0.37		1.50 ± 0.18		4.22 ± 0.83			72h	7.19 ± 1.23		7.35 ± 2.75		10.53 ± 2.86	
ACS6	0h	1.00 ± 0.11		1.00 ± 0.08		1.00 ± 0.10									
	2h	4.40 ± 0.57		5.46 ± 0.21		2.68 ± 0.63									
	24h	1.88 ± 0.18		2.12 ± 0.07		3.09 ± 0.46									
	72h	2.65 ± 0.80		1.86 ± 0.31		1.64 ± 0.17									

In the roots of all genotypes, a strong induction in *OXI1* transcript levels was observed already after 2 h Cu exposure that was sustained after 24 h but decreased again after 72 h exposure (Table 4.2). Downstream targets of OXI1 are members of the mitogen-activated protein kinase (MAPK) protein family (Opdenakker *et al.* 2012b). The *MPK3* transcript levels showed a similar expression pattern as *OXI1* and *MPK4* was also significantly, but transiently upregulated in the roots of all genotypes after 2 h exposure, whereas *MPK6* gene expression was not altered under Cu stress. The expression levels of MAPK-pathway-inducible transcription factors *ZAT7*, *ZAT10* and *ZAT12* were induced in all Cu-exposed roots at all exposure times. Another H_2O_2-signaling gene that influences the expression of *ZAT12* is heat shock factor 21 (*HSF21*), of which the expression was induced in all genotypes and had a similar pattern as *MPK3* expression. As a Cu-responsive transcription factor, *WRKY22* (Opdenakker *et al.* 2012a) was induced in both mutant plants after 2 h and an even higher induction was observed in all genotypes after 24 h.

MAPkinases have also been reported to act upstream of ethylene biosynthesis genes (Joo *et al.* 2008, Han *et al.* 2010). The gene expression of two isoforms of ACC synthase activated by MAPkinases, *ACS2* and *ACS6*, was measured. The transcript levels of *ACS2* were significantly increased and peaked after 24 h Cu exposure in the roots of all genotypes. After 72 h, the expression of *ACS2* was still induced in wildtypes. The expression of *ACS6* was already elevated after 2 h exposure to Cu in the roots of all genotypes and was sustained after 24 h. Moreover, a significantly increased and higher expression was observed in the wildtypes after 72 h Cu exposure compared to *ko-apx2*. Finally, transcript levels of ethylene response factor 1 (*ERF1*), a transcription factor specific in ethylene signal transduction, were increased in the roots of all genotypes after 2 and 24 h, but were significantly higher in wildtypes compared to *ko-apx2* after 72 h Cu exposure. In general, the expression levels of all previously mentioned signaling genes were strongly upregulated after 2 h and some peaked after 24 h exposure to Cu. Additionally, after 72 h exposure to Cu, an elevated expression was still present in wild-type plants for the transcripts of *MPK3*, *HSF21*, *ACS2* and *ACS6* but not in the mutants.

In the leaves (Table 4.3), there was a fast induction after 2 h Cu exposure for several genes, more specifically *OXI1*, *MPK3*, *WRKY22*, *ZAT7*, *ZAT12*, *HSF21,*

ACS2 and *ERF1,* in wild-type and *ko-apx1* plants, which was significantly lower or absent in *ko-apx2* plants. Neither gene expression of *MPK4* nor of *MPK6* was altered under Cu stress. In general, leaves of plants exposed to Cu showed a fast, but transient increase in most of the oxidative signaling genes in wild-type and *ko-apx1*, whereas the induction was postponed and sustained in *ko-apx2* plants (*OXI1*, *MPK3*, *HSF21*, *ACS2*, *ERF1*). The induction of several transcription factors *i.e. ZAT10*, *HSF21* and *ERF1* (all genotypes) and *ZAT7* (*ko-apx1*) was sustained during the entire exposure period (Table 4.3).

Table 4.4. Transcript levels of *CAT2* in roots of 19-days-old *Arabidopsis thaliana* plants exposed to 2 µM $CuSO_4$ for 0, 2, 24 or 72 h. All expression levels are given relatively to the non-exposed wild-type plants (0 µM, 0 h) (1.00 ± SE). Significant expression differences between unexposed genotypes relative to the wildtype are indicated with colored font (induction **green**). Values are mean ± SE of four biological replicates.

ROOT		control		
		wildtype	*ko-apx1*	*ko-apx2*
CAT2	0h	1.00 ± 0.08	**1.45 ± 0.20**	0.98 ± 0.06
	2h	0.82 ± 0.04	**1.33 ± 0.09**	0.88 ± 0.05
	24h	0.96 ± 0.03	**1.61 ± 0.10**	1.02 ± 0.08
	72h	1.02 ± 0.11	**1.76 ± 0.11**	1.04 ± 0.14

4.4. Discussion

To unravel whether APX1 and/or APX2 are involved in Cu-induced oxidative signaling, 19-days-old wildtypes, *ko-apx1* and *ko-apx2 A. thaliana* plants (Col-0 ecotype) were exposed to 2 µM $CuSO_4$ and cellular responses were investigated after 2, 24 and 72 h exposure. After three weeks growth under normal conditions, no visible differences in leaf growth were detected between wild-type and the *ko-apx* mutants. This confirms the study of Suzuki *et al.* (2013) in which under normal circumstances, the single *apx1* (SALK 000249) and *apx2* (SALK 091880) knockout Col-0 plants did not display any visible phenotypes, in contrast to the *ko-apx1* Wassilewskija line that showed a suppressed leaf growth (Pnueli *et al.* 2003, Davletova *et al.* 2005).

External Cu application resulted in Cu accumulation in roots and limited translocation to the leaves (Figure 4.1A,B) leading to root growth inhibition and decreases in fresh weight (Figure 4.2A,B), which is in accordance to Cuypers *et*

al. (2011). Whereas in all genotypes an immediate (2 h) increase in root Cu content was observed and differences in stress levels between wildtypes and *ko-apx* mutants were apparent at this time point. In all genotypes, transcript levels of oxidative stress markers were elevated upon Cu exposure, but lipid peroxidation increased only in the roots of the wildtype (Figure 4.6A), indicating that wildtypes are instantly susceptible to Cu exposure. Concomitantly, an increase in %DW in wild-type plants was observed (Figure 4.3A), which might be an indication of an elevated lignin content, making the cell wall thicker and stiffer (Gomes *et al.* 2011). Previously, it was shown that increased lignification provides the plant sequestering places for excess metals and reduces cellular uptake of these metals from the soil (Chen *et al.* 2002, Cuypers *et al.* 2002, Lin *et al.* 2005, Lequeux *et al.* 2010). In response to the Cu-induced oxidative challenge, all genotypes immediately activated the MAPK signaling cascade at the transcript level (Table 4.2). Opdenakker *et al.* (2012a) demonstrated that the OXI1-MAPK-ZAT cascade is responsive to Cu exposure in *A. thaliana* and is essential to activate gene expression of ROS producing and antioxidative genes (Mittler *et al.* 2004), which was also observed in the present study. For instance, transcript levels of *APX1* were elevated immediately after Cu exposure, suggesting that APX1 plays a central role in oxidative stress responses induced by Cu. Plants lacking APX1 compensate for this loss during Cu stress with an instantly induced *APX2* expression, which was also observed in *A. thaliana ko-apx1* mutants during light and heat stress (Asai *et al.* 2004). In accordance to Smeets *et al.* (2009), our results indicate that also antioxidant metabolites have an important role in both, the defense response and acclimatization of the roots during Cu stress, as genes involved in the biosynthesis of both antioxidant metabolites AsA (*VTC1*) and GSH (*GSH1*, *GSH2*) were induced.

When roots were exposed to Cu for 24 h, the earlier observed stress differences between the wildtypes and mutants disappeared. Morphologically, all genotypes suffered Cu stress to the same extent and showed a decrease in root fresh weight (Figure 4.2A), an increased %DW (Figure 4.3A) and increased lipid peroxidation after 24 h (Figure 4.6A). However, a lower level of TBArm was demonstrated at this time point in *ko-apx2* mutants which were statistically different from wild-type plants. Whereas most signaling genes were strongly and some transiently upregulated at 2 h after Cu exposure, most of the antioxidant

genes were only affected at 24 h. This indicates that the antioxidant defense enzymes are induced upon Cu-induced oxidative signaling in order to cope with and acclimate to Cu stress.

After 72 h Cu exposure, a clear difference was observed between the roots of the wildtype and both *ko-apx* mutants. At this time point, the wildtypes suffered complete growth inhibition together with an increased %DW. In both mutants a small growth recovery and no difference in %DW, as compared to their own controls, was noticed (Figure 4.2A, 4.3A). These results indicate that the *ko-apx* mutants are less sensitive to Cu after prolonged exposure, in contrast to the wild-type roots suffering more stress at this time point. In the study of Suzuki *et al.* (2013), mutant plants lacking APX2 (*A. thaliana*) also showed an increased tolerance to salt and paraquat-induced oxidative stress as compared to the wild-type plants. Although the mutants showed a better acclimation to Cu stress, similar levels of lipid peroxidation were achieved in roots of all genotypes indicating that a different equilibrium is established in the mutants as compared to wild-type plants. This difference was seen in expression levels of oxidative stress markers, antioxidant biosynthesis genes and signaling genes that were still significantly induced in wild-type plants after prolonged Cu exposure, whereas this induction was absent or significantly lower in both mutant plants. The present our results indicate that the induced signaling pathway in wildtypes is due to a different expression pattern of *RBOHD* as compared to the mutants that can give rise to a different signaling, which in turn results in another adaptation response to Cu stress. Previous studies showed a relation between *OXI1* and *RBOHD* in *A. thaliana* exposed to (a)biotic stress (Petersen *et al.* 2009, Takashi *et al*. 2011) as well as under Cu stress (Opdenakker *et al.* 2012a), and they suggested that the production of ROS by NADPH oxidase (RBOHD) can induce the expression of *OXI1* and subsequent downstream components. In wild-type plants, these transcript levels were still induced after prolonged Cu exposure, while no inductions were found in the mutants. MAPkinases (*MPK3* and *MPK6*) have been reported to act upstream of ethylene biosynthesis enzymes, ACS2 and ACS6 (Joo *et al.* 2008, Han *et al.* 2010). Together with the ethylene responsive factor (*ERF1*) a similar gene expression pattern was observed in *ACS2* and *ACS6* that corresponds to the *RBOHD-OXI1* signaling pathway. This indicates that ethylene production and signaling is still present in

the roots of Cu-exposed wild-type plants, whereas it is absent in the *ko-apx* mutants. Copper-induced ethylene synthesis via an increase of ACC synthase activity and the expression of its genes was shown by Sandmann and Böger (1980) and Pell *et al.* (1997). It is known that Cu negatively influences root growth and development (Jiang *et al.* 2000, Lequeux *et al.* 2010, Remans *et al.* 2012), as well as ethylene can in stress conditions increase the rigidity of cell walls through augmented lignification followed by growth inhibition (Enyedi *et al.* 1992). The present study shows that Cu-induced RBOHD signaling might resulted in ethylene synthesis as indicated by the induction of the most important stress induced ethylene biosynthesis genes (Schellingen *et al.* 2014), which eventually could lead to the adverse effect in wild-type plants. This confirms the study of Maksymiec and Krupa (2006a), in which the potential involvement of ethylene and NADPH oxidase in the inhibitory action of Cu in roots of dicotyledons was shown. Furthermore, ethylene stimulates auxin biosynthesis and transport toward the elongation zone of roots, where it activates a local auxin response leading to inhibition of cell elongation (Ruzicka *et al.* 2007). This corresponds to the observed inhibited average lateral root length (Figure 4.4C) in wild-type plants that eventually could lead to a lower root fresh weight.

Although Cu ions were not immediately translocated to the leaves, genes involved in oxidative signaling showed a fast induction in wild-type and *ko-apx1* leaves. In *ko-apx2* plants, the expression of these genes was only significantly induced later (24 h) and lasted longer (still sustained after 72 h), which was not seen in the other two genotypes (Table 4.3). This points towards a muted relay of the signal from the roots. Miller *et al.* (2009) showed that signal propagation requires accumulation of ROS in the extracellular space and rapid expression of ROS-responsive transcripts. Activation of RBOHD along the path of the systemic signal, which enhances ROS production, is essential for signal propagation over short and long distances (Bechtold *et al.* 2008, Miller *et al.* 2009). Whereas the fast induction of *RBOHD* gene expression is present in roots of all genotypes, this is significantly lower in *ko-apx2* leaves in contrast to wild-type plants. The clustering of *OXI1* and *RBOHD* observed in roots, was also previously demonstrated in the leaves of Cu-exposed plants (Opdenakker *et al.* 2012a).

This co-expression was also noticed in the leaves of Cu-exposed *ko-apx2* plants possibly leading to different gene regulation and hence physiological responses. This was clearly reflected in the expression levels of ethylene biosynthesis and signal transduction genes. Eventually, a diminished production of ROS and ethylene could result in a delayed stress response in *ko-apx2* plants showing no upregulations for oxidative markers *OX3* and *OX5* after 2 h. In general, APX2 seems essential for relaying the fast signal transduction from roots to shoots under Cu stress.

When plants were exposed to Cu for 24 h, an increased Cu accumulation was noticed in leaves of all genotypes, concomitantly with a significant increase in lipid peroxidation, no effect on fresh weight and only a significant increase in %DW in wild-type plants (Figure 4.2B, 4.3B, 4.6B). This might indicate a higher susceptibility of wildtypes to Cu exposure in contrast to the *ko-apx* mutants. After 72 h of Cu exposure, this pattern was enforced as there was a growth reduction in all genotypes, but significantly less in both mutants, which was also reflected in the %DW. An increased %DW in leaves may correlates with an inhibition of cell growth as a result of lignification (Lee *et al.* 2007b), and possibly corresponds to closure of the stomata, resulting in a reduction of transpiration preventing further water loss from the leaves (Steuer *et al.* 1988, Lequeux *et al.* 2010). Accumulation of Cu in leaves of young spinach (*Spinacia oleracea*) and oregano (*Origanum vulgare subsp. hirtum*) resulted in a limited rate of transpiration to save water and to retain the internal water balance (Ouzounidou *et al.* 1998, Panou-Filotheou *et al.* 2001).

The observed morphological differences between wildtypes and *ko-apx* mutants probably depend on changes in oxidative signaling and hence different downstream responses. Both antioxidant metabolites AsA and GSH are essential for normal plant development and cell functioning (Foyer and Noctor 2011) as well as for detoxification of Cu-induced oxidative stress (Cuypers *et al.* 2000, Drazkiewicz *et al.* 2003, Jozefczak *et al.* 2012, Bielen *et al.* 2013). After Cu exposure, *ko-apx2* leaves showed an early increase (24 h) in total and reduced AsA that was enhanced in time, which was less pronounced in wildtypes and even less in *ko-apx1* mutants (Table 4.1). Concerning GSH, an increased production of total GSH was only observed after 72 h in both wild-type and *ko-apx2* plants, but with increased GSSG levels in all genotypes. This possibly

indicates that *ko-apx2* mutants are adapting to Cu stress via increased synthesis of antioxidant metabolites, primarily AsA. In the study of Rosa *et al.* (2010), double knockdown (*APX1* and *APX2*) rice plants (*Oryza sativa*) also showed a higher increase in the content of reduced AsA in response to aluminum stress as compared to the wild-type plants. On the other hand, the decreased sensitivity to Cu stress in *ko-apx1* plants seems not to rely on antioxidant metabolites. Cu-exposed *ko-apx1* leaves showed a constitutively higher expression of *ZAT7* during the entire exposure period as compared to Cu-exposed wildtypes. This was in accordance with the study of Suzuki *et al.* (2013), in which *ko-apx1* mutants showed a higher accumulation of *ZAT7* during high light stress as compared to wildtypes. A previous study in which *ZAT7* was constitutively expressed in transgenic *A. thaliana* plants observed that *ZAT7* suppresses a repressor that inhibits the initiation of transcription, thereby removing its inhibition of defense responses, and hence the increase of stress tolerance. Several transcripts encoding defense and regulatory proteins, involved in plant tolerance to salinity stress, were elevated in *ZAT7*-expressing plants, but not in plants expressing *ZAT7* with a deletion or mutation (Ciftzy-Yilmaz *et al.* 2007). A more increased tolerance was shown in *A. thaliana* plants expressing *ZAT7* against paraquat-induced oxidative stress as compared to seedlings of wild-type plants (Rizhsky *et al.* 2004). This indicates that additional components of the oxidative stress signal transduction pathway of *A. thaliana* could be used to enhance the tolerance of plants to oxidative stress. Interestingly, Ciftzy-Yilmaz *et al.* (2007) further compared *ko-apx1* with wild-type plants, and showed that *ko-apx1* plants expressing *ZAT7*, a protein kinase (*WRKY70*) and a protein involved in miRNA transport (*HASTY*), were more tolerant to salinity stress due to the co-expression of these components (Ciftzy-Yilmaz *et al.* 2007).

In conclusion, our results indicate that APX1 and APX2 are involved in cellular signaling during Cu-induced toxicity in roots and leaves of *A. thaliana* (Figure 4.7). In both leaves and roots, *ko-apx* mutants were more tolerant to prolonged Cu exposure, whereas the wild-type plants suffered more stress. In the roots, a more pronounced signaling pathway due to the expression of *RBOHD* in wildtypes give rise to a different signaling, as compared to the mutants, resulting in the synthesis of ethylene and eventually leading to root growth

inhibition. This suggests that APX1 and APX2 are necessary in Cu-induced stimulation of ethylene synthesis. Furthermore, more tolerance against Cu stress in the mutant leaves is achieved by changes in oxidative signaling and hence different downstream responses, which were clearly different between *ko-apx1* and *ko-apx2*. *Ko-apx2* mutants are acclimating to Cu exposure via increased metabolite levels, especially AsA, whereas the presence of *ZAT7* transcripts in *ko-apx1* plants could lead to more tolerance. The results clearly imply a role for both APXs in Cu-induced stress responses. Nevertheless future studies could further elucidate whether these knockouts have a longer survival time than wildtypes when exposed to Cu or whether these APXs are mainly important during short-term exposure.

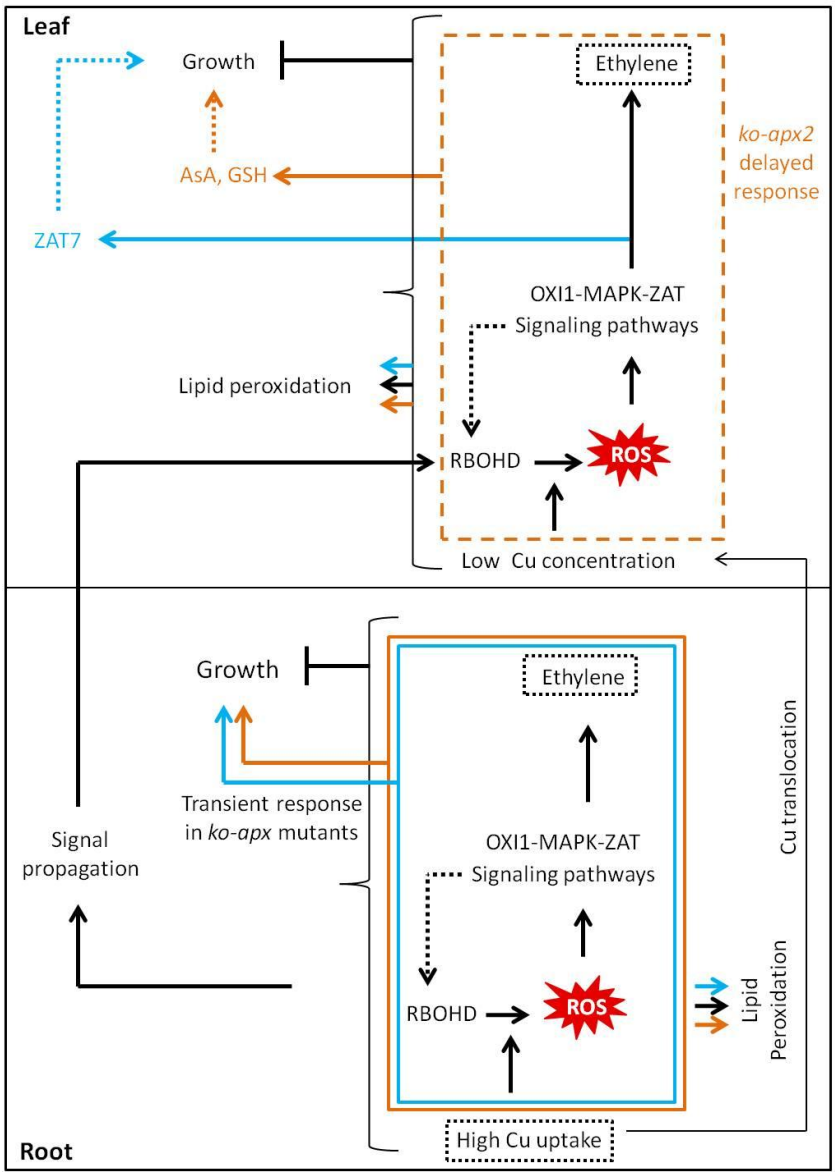

Figure 4.7. Overview of oxidative signaling pathways and defense responses in Cu-exposed *Arabidopsis thaliana* roots and leaves. Black lines represent responses in wildtypes, while different regulated responses in the mutants are represented by blue lines (*ko-apx1*) and orange lines (*ko-apx2*). Dashed lines represent delayed responses. Dotted lines represent pathways found in literature; Cu induces ethylene production in roots and leaves.

CHAPTER 5

A distinct role for APX1 and APX2 in Cd-induced responses in *Arabidopsis thaliana*

An Bielen, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2014). A distinct role for APX1 and APX2 in Cd-induced responses in *Arabidopsis thaliana*. In preparation.

Abstract

In this study, we investigated the importance of ascorbate peroxidase 1 and 2 (APX1 and APX2) in oxidative signaling in *Arabidopsis thaliana* plants exposed to cadmium (Cd). In plants, hydrogen peroxide (H_2O_2) is a major reactive oxygen form that serves as a signaling molecule, which is induced upon exposure to Cd stress. APX1 and APX2 have a high substrate affinity for H_2O_2 and protect the cell against harmful amounts of H_2O_2, while they are also able to regulate H_2O_2 levels for signaling. To investigate the role of APX1/2 in Cd-induced oxidative signaling, wild-type and 2 cytosolic *apx* knockout plants were hydroponically grown and exposed to 5 µM Cd during 24 and 72 h. Our results demonstrate an important role for APX1 in Cd-induced oxidative signaling as *ko-apx1* roots show a suppressed signal transduction resulting in a disturbance of the antioxidative defense system. Furthermore, APX1 is involved in root-to-shoot signaling. A lack of APX2 in plants exposed to Cd results in an altered nutrient profile and AsA metabolism, indicating APX2 plays an essential role in these processes.

Chapter 5

5.1. Introduction

Over the past two centuries, anthropogenic and industrial activities have led to high emissions of cadmium (Cd) into the environment at concentrations significantly exceeding those originating from natural sources (Nriagu 1988, Vangronsveld *et al.* 1995). Cadmium is a non-essential element for plants and is toxic, since 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. Even at low concentrations, Cd disrupts physiological processes in plants, resulting in leaf chlorosis and growth inhibition (Benavides *et al.* 2005, Gallego *et al.* 2012). Whereas Cd is a non-redoxactive metal, it induces oxidative stress at the cellular level, which implies a disturbance of the cellular redox status in favor of the pro-oxidants producing reactive oxygen species (ROS) (Smeets *et al.* 2009, Cuypers *et al.* 2011). The produced ROS are not always harmful for the cell, they can also act as signaling molecules leading to acclimation under stress conditions. Because hydrogen peroxide (H_2O_2) production is an immediate response to increased Cd exposure (Romero-Puertas *et al.* 2004, Cho and Seo 2005), it might be a key molecule that triggers signal transduction events after plant metal exposure (Mithöfer *et al.* 2004, Smeets *et al.* 2008b). In plant cells, the ascorbate (AsA) – glutathione (GSH) cycle plays a central role in the antioxidant defense mechanism to control the delicate balance between H_2O_2 toxicity and H_2O_2 signaling (Foyer and Noctor 2005b). It has been shown that the biosynthesis of these antioxidant metabolites and/or the functioning of the AsA – GSH cycle is disturbed upon metal-induced oxidative stress (Cuypers *et al.* 2000, Aravind and Prasad 2005, Drazkiewicz *et al.* 2010, Mohamed *et al.* 2012, Bielen *et al*. 2013). Ascorbate peroxidase (APX) is the initial enzyme in this cycle in which it reduces H_2O_2 using AsA as an electron donor (Caverzan *et al.* 2012, Bielen *et al.* 2013, Gest *et al.* 2013). In addition, catalases (CATs) are involved in the detoxification of H_2O_2, but whereas their reaction rate is very high, in the presence of AsA their affinity is much lower than APXs. As a consequence, the APXs are involved in the protection against harmful amounts of H_2O_2, as well as in the fine-tuning of H_2O_2 levels for signaling (Mittler 2002, Rizhsky *et al.* 2002, Cuypers *et al.* 2011, Caverzan *et al.* 2012).

Previous studies revealed that cytosolic APXs (APX1 and APX2) are involved in a broad range of stress-induced responses (Yoshimura *et al.* 2000, Rizhsky *et al.*

2004, Caverzan *et al.* 2012). Transcripts of *APX1* are constitutively expressed and under light stress conditions, it was shown that cytosolic APX1 is a regulator of the antioxidant defense mechanism protecting organelles against oxidative stress (Davletova *et al.* 2005, Vanderauwera *et al.* 2011). In contrast, transcript levels of APX2 are almost undetected in many plant tissues under normal conditions, while a strong increase is part of the plant responses to (a)biotic stress (Zimmermann *et al.* 2004, Frank *et al.* 2009). Whereas functional studies on the role of APX2 in plants exposed to different abiotic stress factors [e.g. heat stress (Frank *et al*. 2009), drought stress (Rossel *et al*. 2006), light and salt stress (Suzuki *et al.* 2013)] reveal conflicting results, it is clear that APX2 is involved in the regulation of plant stress responses. However, information on the role of APX2 in the acclimation of plants to metal stress is very limited.

In several studies on plants exposed to Cd stress, a stimulation of the APX enzymatic activity (Schützendübel *et al.* 2001, Aravind and Prasad 2005, Liu *et al.* 2007) as well as an induction at the transcript level for both APX isoforms (Smeets *et al.* 2009, Cuypers *et al.* 2011, Keunen *et al.* 2013) has been observed. Based on the fact (1) that APXs are important in the regulation of cellular H_2O_2 concentrations and (2) that they are affected under Cd stress, we hypothesize that APX1 and APX2 are involved in the regulation of Cd-induced stress responses. To investigate this hypothesis, a functional study in *APX1* knockout (*ko-apx1*) and *APX2* knockout (*ko-apx2*) *A. thaliana* plants exposed to Cd was performed and compared with the responses of wild-type plants.

5.2. Experimental design and methodology

To investigate a possible role for APX1 and APX2 in Cd-induced responses, *A. thaliana* plants (wildtypes, *ko-apx1* and *ko-apx2*) were grown on hydroponics as described previously (Chapter 3, section 3.2). After three weeks the plants were exposed to 5 µM $CdSO_4$ for 24 and 72 h. Besides biomass and metal content, indicators of the cellular redox state were monitored at different biological levels. At transcriptional level, gene expression was analyzed for 1) oxidative stress related genes, 2) genes involved in signal transduction and 3) genes related to ethylene biosynthesis/signaling. At metabolic level, H_2O_2 content, lipid peroxidation, antioxidative enzyme activities and concentration of antioxidative metabolites (AsA and GSH) were investigated.

5.3. Results

5.3.1. Altered element uptake in A. thaliana seedlings under Cd stress

In the roots of all genotypes, the uptake of Cd was significantly increased after 24 h of exposure, compared to the non-exposed roots, and the uptake further increased after 72 h exposure to Cd (Figure 5.1A). Whereas the Cu content of Cd-exposed *ko-apx2* roots was already elevated after 24 h, Cu content increased in all genotypes only after 72 h (Figure 5.1B). No differences were found in the accumulation of Zn in Cd-exposed wild-type and *ko-apx1* roots, while an increase in Zn content was shown in *ko-apx2* roots after 24 and 72 h exposure to Cd (Figure 5.1D).

When 19-days-old *A. thaliana* plants were exposed to 5 µM Cd, 50-60% of the Cd was transported to the leaves. In the leaves, a significant increase in Cd accumulation was observed in all genotypes exposed to Cd for 24 h and had a continuously higher Cd content over time (Figure 5.1A). The Cd-exposed wild-type leaves showed a lower Cu accumulation after 72 h, which was also observed in *ko-apx1* but not in *ko-apx2* plants (Figure 5.1B). The Fe content was elevated in both *ko-apx* mutants only after 72 h exposure to Cd, but not in wild-type plants (Figure 5.1C).

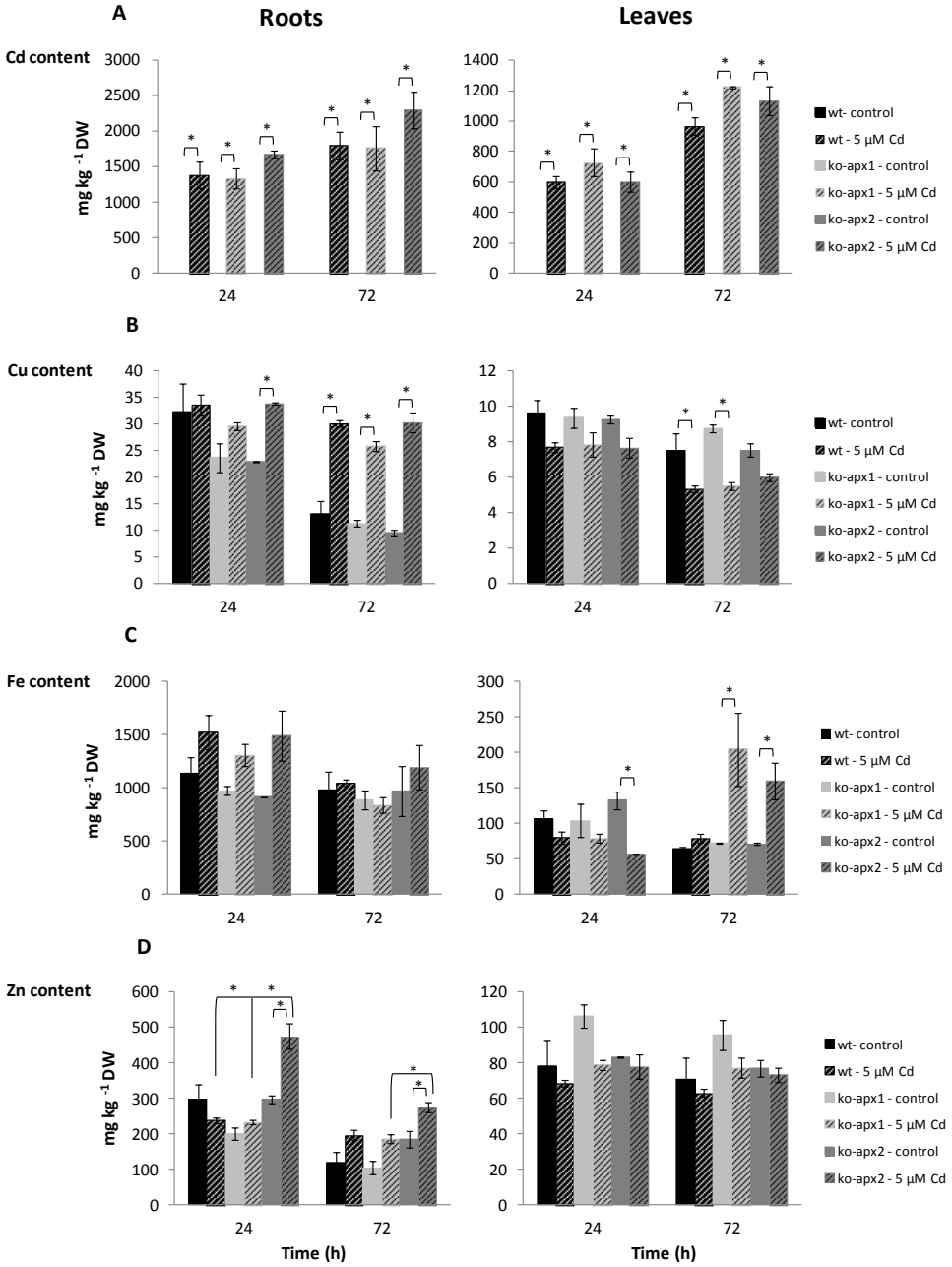

Figure 5.1. The concentrations of Cd content (A), Cu content (B), Fe content (C) and Zn content (D) are expressed as mg kg^{-1} DW in roots (on the left) and leaves (on the right) of 19-days-old *Arabidopsis thaliana* wild-type, *ko-apx1* and *ko-apx2* exposed to 5 µM $CdSO_4$ for 24 and 72 h or grown under control conditions. Values are mean ± SE of four biological replicates. Significant differences within one time point are shown: *p<0.05.

5.3.2. Plant growth

When *A. thaliana* plants were exposed to Cd, a growth reduction was noticed for the roots (Figure 5.2A). All genotypes showed a significantly lower root fresh weight after 72 h exposure to Cd. This was associated with an increase in the dry weight percentage (%DW) in the roots of all genotypes at this time point (Figure 5.2C). Also the leaves of all genotypes exposed to Cd showed a significant increase in %DW after 72 h (Figure 5.2D), whereas no differences in leaf fresh weight were observed (Figure 5.2B).

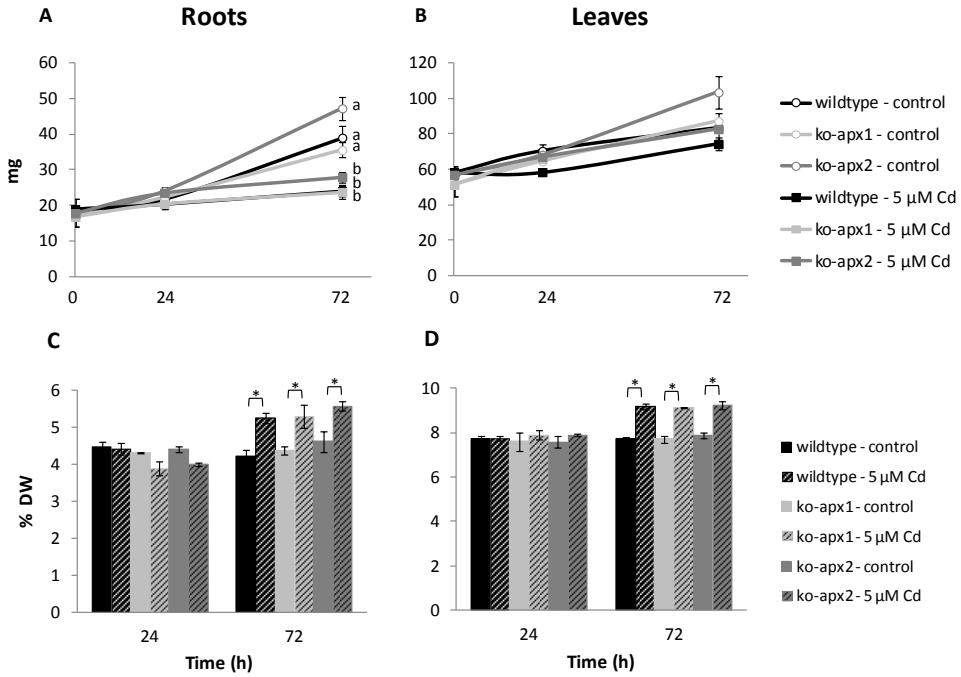

Figure 5.2. Fresh weight (FW, mg) (A,B) and dry weight percentage (%DW) (C,D) in roots (on the left) and leaves (on the right) of 19-days-old *Arabidopsis thaliana* genotypes wild-type, *ko-apx1* and *ko-apx2* plants exposed to 5 µM $CdSO_4$ for 24 and 72 h or grown under control conditions. Values are mean ± SE of ten (A,B) or four (C,D) biological replicates. Significant differences within one time point are shown: (A,B) with different letters ($p<0.05$); (C,D) with * ($p<0.05$).

5.3.3. The cellular redox state of AsA and GSH in A. thaliana ko-apx mutants is differently affected compared to wildtypes after Cd exposure

To tackle Cd-evoked ROS production, plant cells possess an efficient antioxidant defense system in which AsA and GSH function as key antioxidant metabolites to detoxify H_2O_2. Changes in concentration of these metabolites as well as their redox state were investigated in leaves of *A. thaliana* wild-type, *ko-apx1* and *ko-apx2* plants exposed to Cd for 24 and 72 h (Table 5.1).

Table 5.1. Metabolite concentrations are expressed as nmoles g^{-1} FW for GSH and as µmoles g^{-1} FW for AsA in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 or 72 h. Total metabolite concentrations represent both reduced and oxidized forms; the redox state is the ratio between the oxidized and reduced form. Values are mean ± SE of five biological replicates. Significant differences within one time point are indicated with highlighted text p<0.05 induction and repression for an effect of treatment, and significant differences for a genotype effect are shown with different letters (p<0.05).

			wildtype		*ko-apx1*		*ko-apx2*	
			control	5 µM Cd	control	5 µM Cd	control	5 µM Cd
nmoles g^{-1} FW	Total GSH	24h	201.73 ± 10.90	216.75 ± 18.02	203.48 ± 15.44	238.17 ± 9.50	237.84 ± 10.84	232.89 ± 16.01
		72h	179.87 ± 11.03	317.69 ± 13.45 **a**	170.62 ± 10.47	262.22 ± 17.11 **b**	176.67 ± 6.51	322.04 ± 17.84 **a**
	GSH	24h	188.05 ± 10.98	211.72 ± 18.66	190.75 ± 14.80	234.53 ± 9.38	225.26 ± 11.08	230.34 ± 16.43
		72h	169.70 ± 10.66	312.03 ± 13.03 **a**	158.66 ± 10.49	258.28 ± 16.98 **b**	167.61 ± 6.73	316.24 ± 17.25 **a**
	GSSG	24h	13.67 ± 1.75	3.77 ± 0.62	14.71 ± 0.86	3.64 ± 0.61	14.27 ± 1.93	2.55 ± 0.51
		72h	10.16 ± 0.81	5.57 ± 0.63	11.95 ± 0.82	3.94 ± 0.48	9.06 ± 1.09	5.79 ± 1.16
	GSSG/GSH	24h	0.074 ± 0.010	0.020 ± 0.005	0.077 ± 0.006	0.016 ± 0.002	0.065 ± 0.010	0.012 ± 0.003
		72h	0.061 ± 0.005	0.018 ± 0.002	0.078 ± 0.008	0.016 ± 0.002	0.055 ± 0.008	0.018 ± 0.003

			wildtype		*ko-apx1*		*ko-apx2*	
			control	5 µM Cd	control	5 µM Cd	control	5 µM Cd
µmoles g^{-1} FW	Total AsA	24h	4.889 ± 0.671	5.023 ± 0.738	5.287 ± 0.680	5.947 ± 0.827	6.247 ± 0.391	4.612 ± 1.030
		72h	2.273 ± 0.236	3.138 ± 0.612	2.016 ± 0.109	2.380 ± 0.193	2.367 ± 0.444	5.178 ± 1.182
	AsA	24h	2.201 ± 0.217	2.603 ± 0.304	2.249 ± 0.128	2.503 ± 0.352	3.554 ± 0.590	2.854 ± 0.592
		72h	1.396 ± 0.106	1.784 ± 0.238	1.217 ± 0.107	1.534 ± 0.150	1.453 ± 0.113	3.400 ± 0.899
	DHA	24h	2.270 ± 0.617	2.369 ± 0.597	3.038 ± 0.607	3.444 ± 0.639	2.692 ± 0.357	1.758 ± 0.467
		72h	0.877 ± 0.218	1.077 ± 0.275	0.792 ± 0.112	0.826 ± 0.222	0.339 ± 0.050	1.778 ± 0.463
	DHA/ASA	24h	1.151 ± 0.327	0.939 ± 0.238	1.323 ± 0.262	1.422 ± 0.270	0.887 ± 0.224	0.593 ± 0.078
		72h	0.642 ± 0.161	0.410 ± 0.126	0.725 ± 0.200	0.626 ± 0.231	0.230 ± 0.048	0.626 ± 0.136

Under control conditions, no differences in the GSH pool between the genotypes were observed. An increase in total GSH was observed in all genotype leaves exposed to Cd for 72 h, but *ko-apx1* plants showed a significantly lower increase as compared to the other two genotypes. This elevation in total GSH was due to a significant increase in reduced GSH after 72 h in all genotype leaves, but it was once again less pronounced in the leaves of *ko-apx1*. Concerning the

oxidized form (GSSG), significant decreases were noticed in all genotypes after 24 h between the non-exposed and Cd-exposed leaves and this was sustained after 72 h. A similar pattern was observed for the ratio GSSG/GSH in all genotypes.

No differences in AsA contents were observed in wild-type and *ko-apx1* leaves after Cd exposure. However, in the leaves of *ko-apx2*, significant increases were found in total AsA, reduced AsA and DHA after 72 h exposure to Cd, indicating that this mutant needed AsA in response to Cd stress.

5.3.4. The level of ROS production in ko-apx mutants differs from the wildtypes

Accumulation of H_2O_2 was visualized by DAB staining in leaves of wildtypes and *ko-apx* mutants after Cd exposure for 24 and 72 h (Figure 5.3). The H_2O_2 accumulation in the control group was negligible and no visible genotype differences were observed. After exposure to Cd for 24 h an increase in H_2O_2 production was observed in all genotype leaves compared to their non-exposed leaves. Nevertheless, the intensity of brown staining was less pronounced in *ko-apx1* compared to the other two genotypes. The difference between the non-exposed and Cd-exposed group after 24 h was still observed after 72 h in all genotypes. Once again the staining was less pronounced in the leaves of *ko-apx1* mutant compared to wild-type and *ko-apx2* plants.

Lipid peroxidation was investigated by determining tissue levels of TBA-reactive metabolites (TBArm) to compare the oxidative damage between the different genotypes when exposed to Cd. No significant differences were observed between the non-exposed and Cd-exposed plants neither in roots nor in leaves (data not shown).

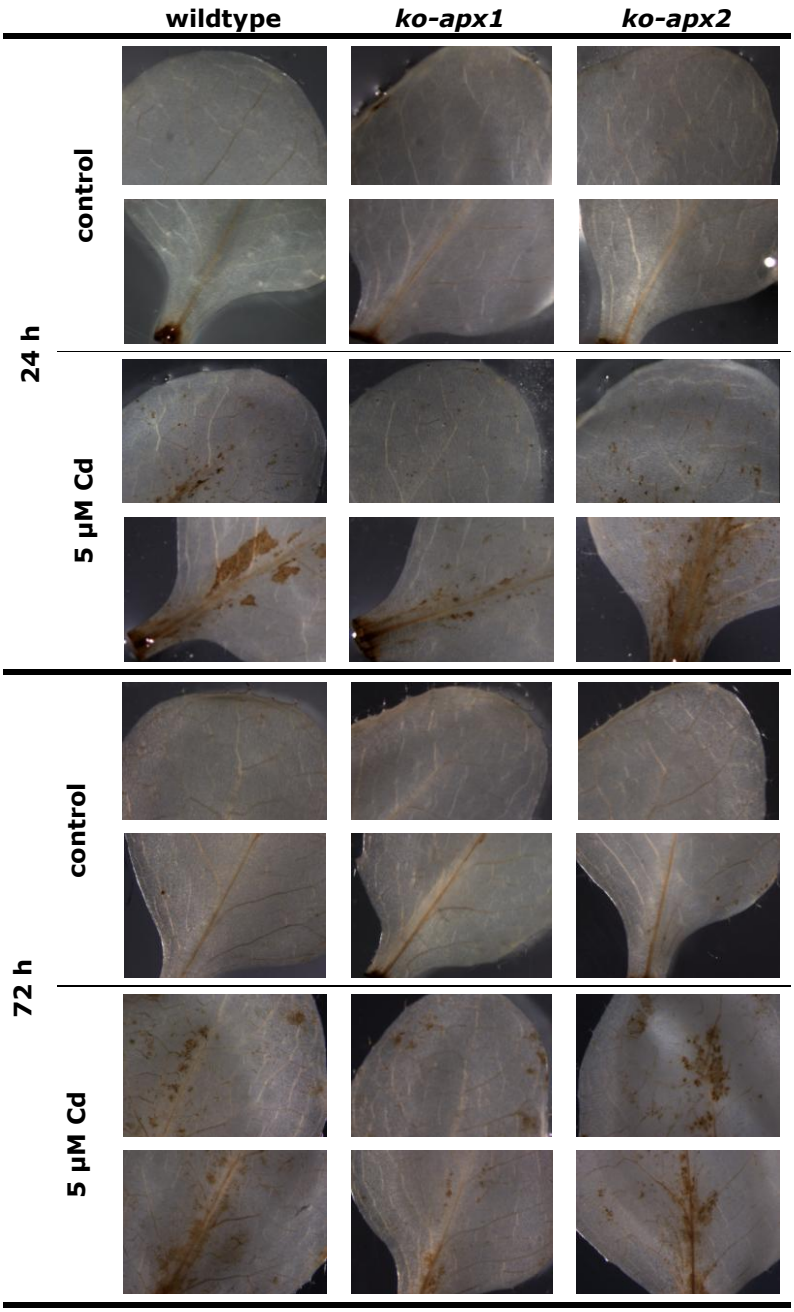

Figure 5.3. Leaves of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ for 24 and 72 h were stained with 3,3'-diaminobenzidine (DAB) to visualize the presence of H_2O_2. Brown spots were found in all genotypes after exposure to Cd, but it was less pronounced in *ko-apx1* leaves.

5.3.5. Alterations in the gene expression network of A. thaliana during Cd stress

5.3.5.1. Antioxidative gene expression

A number of antioxidative genes were measured in roots and leaves of all genotypes. These included different isoforms of superoxide dismutase (SOD), CAT as well as the cytosolic isoforms of APX. Furthermore, genes involved in the AsA-GSH cycle, like dehydroascorbate dehydrogenase (*DHAR*) and glutathione reductase (*GR*), were also analyzed.

In roots (Table 5.2), the expression of *FSD1* was significantly upregulated after 24 h Cd exposure in all genotypes and was still induced after 72 h. In contrast, the transcript levels of *CSD1* and *CSD2* were significantly decreased when exposed to Cd for 72 h in all genotypes, which was a consequence of the upregulated expression and posttranscriptional regulation of *miRNAs* after Cd exposure. Figure 5.4A is shown to gain more insight in the actions of the different *CAT* isoforms in roots. The most abundant isoform in the roots of all genotypes was *CAT2*, which was constitutively higher in *ko-apx1* roots (Figure 5.4A). Only *CAT1* of the CAT gene family showed an increased transcript level in all genotypes after 24 h exposure to Cd, and the expression was significantly higher in *ko-apx1* as compared to wildtypes. Only *ko-apx2* plants showed a significantly increased transcript level of *CAT3* after 72 h (Table 5.2).

In leaves (Table 5.3), the expression pattern of all antioxidative genes peaked after 24 h Cd exposure in all genotypes. The expression of *CSD1* was increased in all genotypes after 24 h exposure and a significantly higher expression was observed in *ko-apx1* compared to wildtype and *ko-apx2*. After 72 h exposure to Cd the transcript levels of *CSD1* and *CSD2* were decreased, which was due to the upregulation of *miRNA*s in all genotypes (Sunkar *et al.* 2006). The three *CAT* isoforms were also investigated in the leaves (Figure 5.4B). Similar as in roots, the most abundant isoform in leaves was also *CAT2*, of which the transcripts were decreased in all genotype leaves exposed to Cd for 24 h with a significantly lower transcript level for *ko-apx1* leaves. The decreased expression of *CAT2* was sustained after 72 h in all genotypes. As shown in Table 5.3, the expression of *CAT1* was increased after 24 h Cd exposure in all genotypes, with a significantly

Table 5.2. Transcript levels in roots of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 or 72 h. Fold changes in expression levels within a genotype and time point are relative to the non-exposed plants (0 µM) (1.00 ± SE). A significant effect of treatment (relative to their own controls) is indicated with highlighted text: p<0.05 induction , repression ; p<0.01 induction , repression ; p<0.001 induction , repression . Significant interaction effects (genotype*treatment) within a time point are represented by different letters. Values are mean ± SE of four biological replicates.

ROOT		wildtype			*ko-apx1*			*ko-apx2*		
		control	5 µM Cd		control	5 µM Cd		control	5 µM Cd	
Genes coding for antioxidant enzymes										
CSD1	24h	1.00 ± 0.04	1.00 ± 0.06		1.00 ± 0.04	0.96 ± 0.08		1.00 ± 0.07	0.80 ± 0.04	
	72h	1.00 ± 0.12	0.47 ± 0.01		1.00 ± 0.05	0.44 ± 0.02		1.00 ± 0.11	0.45 ± 0.03	
CSD2	24h	1.00 ± 0.04	0.91 ± 0.02	a	1.00 ± 0.04	1.00 ± 0.04	a	1.00 ± 0.03	0.75 ± 0.05	b
	72h	1.00 ± 0.08	0.55 ± 0.03		1.00 ± 0.02	0.57 ± 0.02		1.00 ± 0.06	0.55 ± 0.05	
miRNA398a	24h	1.00 ± 0.05	6.73 ± 0.70		1.00 ± 0.35	4.59 ± 1.36		1.00 ± 0.23	4.98 ± 1.19	
	72h	1.00 ± 0.58	0.32 ± 0.05		1.00 ± 0.41	0.40 ± 0.05		1.00 ± 0.25	0.16 ± 0.04	
miRNA398b	24h	1.00 ± 0.09	2.58 ± 0.21		1.00 ± 0.07	3.15 ± 0.04		1.00 ± 0.12	3.24 ± 0.26	
	72h	1.00 ± 0.22	2.88 ± 0.23		1.00 ± 0.05	2.11 ± 0.23		1.00 ± 0.13	2.43 ± 0.18	
miRNA398c	24h	1.00 ± 0.14	33.79 ± 8.45		1.00 ± 0.33	27.89 ± 1.61		1.00 ± 0.28	40.89 ± 5.99	
	72h	1.00 ± 0.40	15.56 ± 2.54		1.00 ± 0.24	7.40 ± 0.97		1.00 ± 0.31	14.47 ± 3.07	
FSD1	24h	1.00 ± 0.19	9.96 ± 2.01		1.00 ± 0.31	12.80 ± 2.23		1.00 ± 0.21	24.84 ± 3.18	
	72h	1.00 ± 0.49	6.66 ± 1.09		1.00 ± 0.23	11.20 ± 0.29		1.00 ± 0.27	10.56 ± 1.31	
CAT1	24h	1.00 ± 0.04	1.40 ± 0.07	a	1.00 ± 0.04	1.82 ± 0.12	b	1.00 ± 0.03	1.48 ± 0.18	ab
	72h	1.00 ± 0.03	1.09 ± 0.02		1.00 ± 0.08	1.03 ± 0.06		1.00 ± 0.09	1.04 ± 0.08	
CAT2	24h	1.00 ± 0.03	0.85 ± 0.12		1.00 ± 0.06	1.14 ± 0.03		1.00 ± 0.14	0.88 ± 0.06	
	72h	1.00 ± 0.03	0.66 ± 0.06		1.00 ± 0.06	0.77 ± 0.07		1.00 ± 0.06	0.85 ± 0.02	
CAT3	24h	1.00 ± 0.07	0.89 ± 0.13	a	1.00 ± 0.07	1.46 ± 0.11	b	1.00 ± 0.05	0.91 ± 0.07	a
	72h	1.00 ± 0.13	1.16 ± 0.03		1.00 ± 0.09	1.52 ± 0.12		1.00 ± 0.05	1.88 ± 0.27	
APX1	24h	1.00 ± 0.02	0.76 ± 0.02					1.00 ± 0.06	0.73 ± 0.03	
	72h	1.00 ± 0.03	0.73 ± 0.03					1.00 ± 0.04	0.85 ± 0.09	
APX2	24h	1.00 ± 0.10	1.59 ± 0.33		1.00 ± 0.02	1.87 ± 0.67				
	72h	1.00 ± 0.19	0.68 ± 0.07		1.00 ± 0.10	0.60 ± 0.04				
DHAR1	24h	1.00 ± 0.03	1.06 ± 0.04		1.00 ± 0.02	1.18 ± 0.04		1.00 ± 0.06	0.98 ± 0.07	
	72h	1.00 ± 0.03	0.99 ± 0.05		1.00 ± 0.03	1.02 ± 0.07		1.00 ± 0.05	1.18 ± 0.04	
GR1	24h	1.00 ± 0.07	1.19 ± 0.08		1.00 ± 0.02	1.24 ± 0.05		1.00 ± 0.08	1.01 ± 0.04	
	72h	1.00 ± 0.10	1.21 ± 0.08		1.00 ± 0.06	1.07 ± 0.01		1.00 ± 0.05	1.19 ± 0.07	
GR2	24h	1.00 ± 0.06	1.09 ± 0.07	a	1.00 ± 0.01	1.19 ± 0.02	a	1.00 ± 0.09	0.83 ± 0.02	b
	72h	1.00 ± 0.06	0.87 ± 0.05		1.00 ± 0.01	0.94 ± 0.03		1.00 ± 0.02	0.89 ± 0.03	
Genes coding for ROS production										
RBOHD	24h	1.00 ± 0.15	1.94 ± 0.19		1.00 ± 0.23	2.00 ± 0.12		1.00 ± 0.12	2.20 ± 0.09	
	72h	1.00 ± 0.05	2.32 ± 0.22	a	1.00 ± 0.09	1.46 ± 0.17	b	1.00 ± 0.13	2.30 ± 0.26	a
Genes coding for regulatory enzymes										
OXI1	24h	1.00 ± 0.07	1.57 ± 0.20	ab	1.00 ± 0.08	1.43 ± 0.11	a	1.00 ± 0.12	2.28 ± 0.17	b
	72h	1.00 ± 0.02	3.02 ± 0.44	a	1.00 ± 0.07	0.98 ± 0.16	b	1.00 ± 0.18	1.39 ± 0.12	b
MPK3	24h	1.00 ± 0.04	2.26 ± 0.14		1.00 ± 0.03	2.02 ± 0.03		1.00 ± 0.09	2.04 ± 0.15	
	72h	1.00 ± 0.02	2.28 ± 0.10	a	1.00 ± 0.07	1.64 ± 0.12	b	1.00 ± 0.06	1.85 ± 0.11	ab
MPK4	24h	1.00 ± 0.04	1.36 ± 0.07		1.00 ± 0.02	1.45 ± 0.07		1.00 ± 0.07	1.25 ± 0.06	
	72h	1.00 ± 0.05	1.29 ± 0.05		1.00 ± 0.02	1.10 ± 0.02		1.00 ± 0.06	1.12 ± 0.11	
MPK6	24h	1.00 ± 0.04	1.18 ± 0.01		1.00 ± 0.01	1.11 ± 0.04		1.00 ± 0.05	1.06 ± 0.04	
	72h	1.00 ± 0.03	0.89 ± 0.04	ab	1.00 ± 0.04	0.77 ± 0.01	a	1.00 ± 0.04	1.00 ± 0.03	b
WRKY22	24h	1.00 ± 0.02	0.88 ± 0.08		1.00 ± 0.17	0.68 ± 0.07		1.00 ± 0.10	0.73 ± 0.07	
	72h	1.00 ± 0.08	0.70 ± 0.04	a	1.00 ± 0.12	0.44 ± 0.03	b	1.00 ± 0.18	0.71 ± 0.05	a
ZAT7	24h	1.00 ± 0.26	10.14 ± 2.09		1.00 ± 0.13	7.37 ± 2.37		1.00 ± 0.15	14.76 ± 2.59	
	72h	1.00 ± 0.39	17.16 ± 5.52		1.00 ± 0.06	7.98 ± 1.60		1.00 ± 0.31	23.47 ± 9.97	
ZAT10	24h	1.00 ± 0.04	4.78 ± 0.79		1.00 ± 0.21	2.77 ± 0.35		1.00 ± 0.04	4.11 ± 0.37	
	72h	1.00 ± 0.23	7.01 ± 1.06	a	1.00 ± 0.14	1.72 ± 0.32	b	1.00 ± 0.20	5.28 ± 1.38	a
ZAT12	24h	1.00 ± 0.11	2.40 ± 0.52	a	1.00 ± 0.19	2.75 ± 0.62	a	1.00 ± 0.07	5.53 ± 0.38	b
	72h	1.00 ± 0.31	4.42 ± 0.54	a	1.00 ± 0.17	1.56 ± 0.36	b	1.00 ± 0.17	3.07 ± 0.60	ab
HSF21	24h	1.00 ± 0.05	1.42 ± 0.09	a	1.00 ± 0.09	1.93 ± 0.16	b	1.00 ± 0.06	1.91 ± 0.08	b
	72h	1.00 ± 0.02	1.85 ± 0.18	a	1.00 ± 0.07	1.21 ± 0.11	b	1.00 ± 0.08	2.05 ± 0.17	a
Genes involved in the ethylene biosynthesis										
ACS2	24h	1.00 ± 0.06	2.46 ± 0.62	ab	1.00 ± 0.08	1.67 ± 0.51	a	1.00 ± 0.07	4.30 ± 0.86	b
	72h	1.00 ± 0.14	3.86 ± 1.17	a	1.00 ± 0.09	1.20 ± 0.30	b	1.00 ± 0.01	2.07 ± 0.43	ab
ACS6	24h	1.00 ± 0.08	3.95 ± 0.54		1.00 ± 0.11	2.77 ± 0.02		1.00 ± 0.12	4.42 ± 0.01	
	72h	1.00 ± 0.16	3.84 ± 0.60	a	1.00 ± 0.06	1.60 ± 0.23	b	1.00 ± 0.06	2.51 ± 0.29	ab

higher expression level in *ko-apx1* leaves. The third CAT isoform, *CAT3*, was only upregulated in mutants after 72 h exposure to Cd. The transcript level of *APX1* was induced after 24 h in wildtypes and *ko-apx2*, while after 72 h a decreased level was found only in the wildtypes. The transcript levels of *GR1* were, such as *CAT1*, increased in all genotypes with a significantly higher expression in *ko-apx1* after 24 h Cd exposure, and the expression of *GR1* was sustained after 72 h in both mutants. In all genotypes, a decreased expression was found for *DHAR1* and *GR2* after 24 h Cd exposure and the expression was, such as *CAT2*, significantly lower in *ko-apx1* compared to the other genotypes. The transcript level of *DHAR1* was sustained after 72 h in both mutants but not in wildtypes. In general, in leaves a more pronounced expression level was observed in *ko-apx1* plants after 24 h Cd exposure compared to wildtypes and *ko-apx2*. After 72 h, the transcript level of some antioxidative genes was still sustained in both mutants but not in wildtypes.

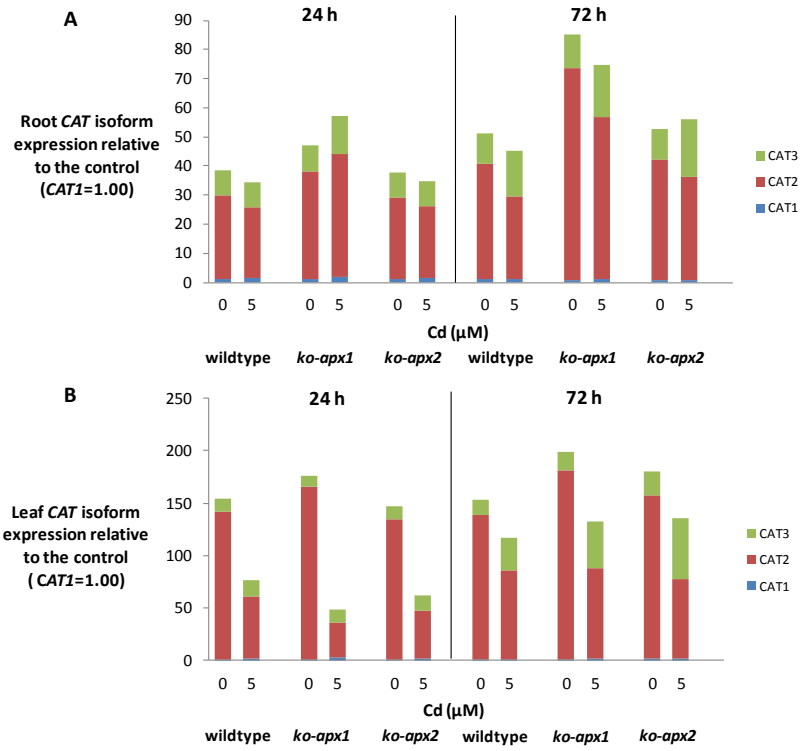

Figure 5.4. Exposure to Cd has an effect on the relative abundance of the CAT gene family members in *Arabidopsis thaliana* plants. Plants were exposed to 5 $CdSO_4$ for 24 and 72 h. Data are given as the mean abundance of at least 4 biological replicates relative to the control (24 h, 0 µM) with the abundance of the lowest expressed family member set at 1.00. The relative abundance of CAT gene family members in the roots (A) and in the leaves (B).

5.3.5.2. Genes involved in oxidative signaling

In order to gather more information concerning the signaling in the different *A. thaliana* genotypes after Cd stress, the gene expression of ROS producing NADPH oxidase (RBOHD), H_2O_2-sensitive serine/threonine kinase OXI1 (oxidative signal-inducible 1), downstream mitogen-activated protein kinase (MAPK) protein family (Opdenakker *et al.* 2012b), and transcription factors was analyzed.

In roots (Table 5.2), an increase was observed in the expression of the ROS-producing gene *RBOHD* after 24 h Cd exposure in all genotypes, but after 72 h the increased expression was only sustained and significantly higher in wildtype and *ko-apx2*. Wild-type and *ko-apx2* roots showed an induced transcript level of *OXI1* after 24 h Cd exposure, which was significantly higher in *ko-apx2* as compared to *ko-apx1*. Only in wildtypes the expression level of *OXI1* was sustained and significantly higher compared to both *ko-apx* mutant roots. Downstream targets of OXI1, *i.e. MPK3*, *MPK4* and *MPK6* were upregulated in roots of wildtypes after 24 h Cd exposure, which was sustained after 72 h except for *MPK6*. Increased expression levels of *MPK3* and *MPK4* were also found in both mutants after 24 h, but only the transcripts of *MPK3* remained upregulated after 72 h with a significantly lower expression in *ko-apx1* compared to the wildtypes. The expression of MAPK-pathway-inducible transcription factors *ZAT7*, *ZAT10* and *ZAT12* had a similar expression pattern as *MPK3* and *MPK4*, and were induced in all Cd-exposed roots after 24 h, and remained significantly increased after 72 h for *ZAT7* in all genotypes, but only in wild-type and *ko-apx2* roots for *ZAT10* and *ZAT12* expression. Another H_2O_2-signaling gene that influences the expression of *ZAT12* is heat shock factor 21 (*HSF21*), of which the expression pattern was similar to *ZAT12* expression.

Table 5.3. Transcript levels in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 or 72 h. Fold changes in expression levels within a genotype and time point are relative to the non-exposed plants (0 µM) (1.00 ± SE). A significant effect of treatment (relative to their own controls) is indicated with highlighted text: p<0.05 induction, repression; p<0.01 induction, repression; p<0.001 induction, repression. Significant interaction effects (genotype*treatment) within a time point are represented by different letters. Values are mean ± SE of four biological replicates.

LEAF		wildtype			*ko-apx1*			*ko-apx2*		
		control	5 µM Cd		control	5 µM Cd		control	5 µM Cd	
Genes coding for antioxidant enzymes										
CSD1	24h	1.00 ± 0.04	1.63 ± 0.12	a	1.00 ± 0.07	2.42 ± 0.23	b	1.00 ± 0.03	1.67 ± 0.23	a
	72h	1.00 ± 0.03	0.42 ± 0.02	a	1.00 ± 0.12	0.62 ± 0.03	b	1.00 ± 0.08	0.44 ± 0.04	a
CSD2	24h	1.00 ± 0.06	0.76 ± 0.10		1.00 ± 0.11	0.59 ± 0.07		1.00 ± 0.02	0.58 ± 0.07	
	72h	1.00 ± 0.02	0.13 ± 0.02		1.00 ± 0.11	0.15 ± 0.01		1.00 ± 0.12	0.12 ± 0.02	
miRNA398a	24h	1.00 ± 0.07	249.11 ± 54.75		1.00 ± 0.26	255.53 ± 51.87		1.00 ± 0.07	401.81 ± 55.55	
	72h	1.00 ± 0.27	11.89 ± 1.57		1.00 ± 0.25	35.56 ± 6.86		1.00 ± 0.25	17.70 ± 2.61	
miRNA398b	24h	1.00 ± 0.09	3.29 ± 0.54		1.00 ± 0.35	2.44 ± 0.73		1.00 ± 0.09	5.02 ± 0.44	
	72h	1.00 ± 0.30	1.65 ± 0.15		1.00 ± 0.11	1.21 ± 0.19		1.00 ± 0.25	1.79 ± 0.35	
miRNA398c	24h	1.00 ± 0.11	10.04 ± 2.06		1.00 ± 0.45	4.19 ± 1.19		1.00 ± 0.14	12.59 ± 2.15	
	72h	1.00 ± 0.36	3.75 ± 0.57		1.00 ± 0.30	4.17 ± 0.71		1.00 ± 0.23	5.57 ± 1.42	
FSD1	24h	1.00 ± 0.08	3.76 ± 1.20		1.00 ± 0.42	0.60 ± 0.27		1.00 ± 0.25	4.17 ± 1.35	
	72h	1.00 ± 0.32	1.06 ± 0.26		1.00 ± 0.23	0.61 ± 0.03		1.00 ± 0.30	1.94 ± 0.23	
CAT1	24h	1.00 ± 0.05	1.68 ± 0.05	a	1.00 ± 0.11	2.44 ± 0.12	b	1.00 ± 0.05	1.82 ± 0.05	ab
	72h	1.00 ± 0.07	0.85 ± 0.12		1.00 ± 0.03	1.21 ± 0.20		1.00 ± 0.07	1.28 ± 0.15	
CAT2	24h	1.00 ± 0.06	0.42 ± 0.03	a	1.00 ± 0.13	0.20 ± 0.01	b	1.00 ± 0.04	0.34 ± 0.05	a
	72h	1.00 ± 0.02	0.61 ± 0.08		1.00 ± 0.05	0.45 ± 0.02		1.00 ± 0.11	0.52 ± 0.01	
CAT3	24h	1.00 ± 0.04	1.25 ± 0.03		1.00 ± 0.12	1.18 ± 0.13		1.00 ± 0.08	1.27 ± 0.07	
	72h	1.00 ± 0.13	1.41 ± 0.13		1.00 ± 0.21	2.20 ± 0.26		1.00 ± 0.19	2.53 ± 0.29	
APX1	24h	1.00 ± 0.04	1.43 ± 0.11					1.00 ± 0.06	1.52 ± 0.11	
	72h	1.00 ± 0.06	0.70 ± 0.08	a				1.00 ± 0.02	1.07 ± 0.10	b
APX2	24h	1.00 ± 0.16	2.73 ± 0.45		1.00 ± 0.15	3.67 ± 0.44				
	72h	1.00 ± 0.16	0.79 ± 0.15		1.00 ± 0.13	1.10 ± 0.15				
DHAR1	24h	1.00 ± 0.07	0.58 ± 0.06	a	1.00 ± 0.06	0.36 ± 0.05	b	1.00 ± 0.06	0.54 ± 0.02	a
	72h	1.00 ± 0.11	0.71 ± 0.05		1.00 ± 0.01	0.53 ± 0.04		1.00 ± 0.17	0.59 ± 0.05	
GR1	24h	1.00 ± 0.02	2.64 ± 0.17	a	1.00 ± 0.03	3.13 ± 0.16	b	1.00 ± 0.03	2.42 ± 0.13	a
	72h	1.00 ± 0.08	1.17 ± 0.08		1.00 ± 0.04	1.49 ± 0.15		1.00 ± 0.08	1.47 ± 0.09	
GR2	24h	1.00 ± 0.04	0.69 ± 0.05	a	1.00 ± 0.08	0.49 ± 0.04	b	1.00 ± 0.04	0.57 ± 0.05	ab
	72h	1.00 ± 0.03	0.76 ± 0.07		1.00 ± 0.10	0.80 ± 0.04		1.00 ± 0.14	0.84 ± 0.04	
Genes coding for ROS production										
RBOHD	24h	1.00 ± 0.02	3.63 ± 0.09	a	1.00 ± 0.04	3.56 ± 0.32	a	1.00 ± 0.10	2.19 ± 0.26	b
	72h	1.00 ± 0.18	1.59 ± 0.07		1.00 ± 0.05	2.58 ± 0.22		1.00 ± 0.18	2.21 ± 0.18	
Genes coding for regulatory enzymes										
OXI1	24h	1.00 ± 0.02	88.36 ± 9.44	a	1.00 ± 0.12	114.26 ± 13.36	a	1.00 ± 0.13	56.61 ± 9.47	b
	72h	1.00 ± 0.44	10.75 ± 3.57		1.00 ± 0.29	39.28 ± 10.99		1.00 ± 0.32	20.53 ± 3.39	
MPK3	24h	1.00 ± 0.10	6.77 ± 0.32		1.00 ± 0.02	6.93 ± 1.01		1.00 ± 0.11	6.47 ± 0.29	
	72h	1.00 ± 0.24	2.00 ± 0.14		1.00 ± 0.24	2.42 ± 0.10		1.00 ± 0.26	1.34 ± 0.05	
MPK4	24h	1.00 ± 0.04	2.62 ± 0.12		1.00 ± 0.02	2.95 ± 0.19		1.00 ± 0.08	2.84 ± 0.17	
	72h	1.00 ± 0.17	1.48 ± 0.09		1.00 ± 0.12	1.73 ± 0.13		1.00 ± 0.05	1.09 ± 0.06	
MPK6	24h	1.00 ± 0.02	1.97 ± 0.09	a	1.00 ± 0.03	2.80 ± 0.23	b	1.00 ± 0.04	1.94 ± 0.15	a
	72h	1.00 ± 0.14	1.04 ± 0.05		1.00 ± 0.03	0.93 ± 0.05		1.00 ± 0.01	0.98 ± 0.02	
WRKY22	24h	1.00 ± 0.04	1.57 ± 0.12	a	1.00 ± 0.05	2.12 ± 0.16	b	1.00 ± 0.07	1.42 ± 0.15	a
	72h	1.00 ± 0.15	0.70 ± 0.07		1.00 ± 0.04	1.12 ± 0.15		1.00 ± 0.19	1.20 ± 0.05	
ZAT7	24h	1.00 ± 0.31	18.27 ± 0.94	a	1.00 ± 0.15	33.41 ± 2.95	b	1.00 ± 0.52	6.51 ± 0.62	ac
	72h	1.00 ± 0.30	3.27 ± 1.16		1.00 ± 0.65	7.20 ± 0.97		1.00 ± 0.44	2.74 ± 0.30	
ZAT10	24h	1.00 ± 0.12	14.85 ± 0.47	a	1.00 ± 0.09	21.57 ± 1.53	b	1.00 ± 0.07	13.38 ± 1.98	a
	72h	1.00 ± 0.32	0.44 ± 0.09	a	1.00 ± 0.23	4.21 ± 0.83	b	1.00 ± 0.28	3.05 ± 0.36	ab
ZAT12	24h	1.00 ± 0.09	22.33 ± 3.62	a	1.00 ± 0.17	49.37 ± 4.69	b	1.00 ± 0.10	15.02 ± 2.09	a
	72h	1.00 ± 0.26	1.80 ± 0.65	a	1.00 ± 0.38	13.98 ± 4.02	b	1.00 ± 0.30	8.92 ± 1.74	b
HSF21	24h	1.00 ± 0.05	19.86 ± 0.92		1.00 ± 0.04	16.21 ± 1.90		1.00 ± 0.10	15.62 ± 1.55	
	72h	1.00 ± 0.35	2.01 ± 0.23	a	1.00 ± 0.23	6.05 ± 0.56	b	1.00 ± 0.30	2.75 ± 0.20	ab
Genes involved in the ethylene biosynthesis										
ACS2	24h	1.00 ± 0.37	231.48 ± 28.24	a	1.00 ± 0.34	1378.68 ± 141.11	b	1.00 ± 0.36	452.27 ± 85.39	a
	72h	1.00 ± 0.50	9.07 ± 3.51	a	1.00 ± 0.71	168.34 ± 56.16	b	1.00 ± 0.42	37.01 ± 9.51	ab
ACS6	24h	1.00 ± 0.07	21.93 ± 0.73		1.00 ± 0.04	18.90 ± 2.52		1.00 ± 0.07	15.65 ± 1.23	
	72h	1.00 ± 0.22	1.31 ± 0.38	a	1.00 ± 0.22	3.85 ± 0.63	b	1.00 ± 0.13	3.18 ± 0.29	b

In addition, two isoforms of ACC synthase (involved in ethylene biosynthesis) ACS2 and ACS6 responsive to Cd (Schellingen *et al.* 2014) and activated by MAPkinases (Joo *et al.* 2008, Han *et al.* 2010) were measured. The transcripts of *ACS2* were upregulated in wildtypes and *ko-apx2* after 24 h, and were significantly higher in the latter as compared to *ko-apx1*. Only in wild-type roots the expression level of *ACS2* was still induced after 72 h Cd exposure. The transcript level of *ACS6* was induced in all genotypes after 24 h and was sustained in wildtypes and *ko-apx2* after 72 h Cd exposure. In general, roots exposed to Cd for 24 h showed an induction in previously mentioned signaling genes in most genotypes. After 72 h exposure to Cd, an elevated expression was still present in wild-type and *ko-apx2* plants, while a significantly lower or no significant difference in expression was found for the *ko-apx1* mutant.

In leaves (Table 5.3), inductions in gene expression involved in signaling were observed and reached a maximum after 24 h Cd exposure, a pattern that was found in all genotypes. The transcripts of *RBOHD* were increased in all genotypes after 24 h, but with a significantly lower expression in *ko-apx2* leaves. Similarly, the expression of *OXI1* was upregulated in all Cd-exposed genotypes after 24 h, again with a significantly lower increase in *ko-apx2* leaves. In addition, the upregulations of *RBOHD* and *OXI1* were sustained after 72 h Cd exposure especially in both mutants. A significantly higher expression in the leaves of *ko-apx1*, in comparison with both other genotypes, was found after 24 h Cd exposure in the transcript levels of *MPK6, WRKY22*, *ZAT7*, *ZAT10*, *ZAT12* and *ACS2.* Similar to *RBOHD* transcript levels, the expression of *ZAT10*, *ZAT12*, *HSF21*, *ACS2* and *ACS6* remained significantly induced in both mutants after 72 h Cd exposure in contrast to the wild-type plants. In general, a higher Cd-induced response was found for *ko-apx1* leaves after 24 h exposure, while a lower Cd-induced response was observed in *ko-apx2* leaves for *RBOHD* and *OXI1*. After 72 h, the transcript levels of the signaling genes were still induced in both mutants, but not in wild-type plants.

5.3.6. Alterations in enzyme activities in wildtypes versus ko-apx mutants after exposure to Cd stress

In addition to the gene expression measurements, activities of antioxidative enzymes were measured in roots and leaves of 19-days-old *A. thaliana* plants exposed to Cd for 24 and 72 h (Table 5.4). In roots, under control conditions, the activity of APX was significantly lower for *ko-apx1*, compared to the other genotypes. An increased activity of APX, as well as SOD, was found in wildtype and *ko-apx2* after 72 h Cd exposure, which was not observed in *ko-apx1* plants. Furthermore, after Cd exposure for 24 h, an enhancement was observed in the GAPDH activity, which was significant for the wild-type roots.

In leaves, APX activity showed a slight decrease in both mutants after Cd exposure for 24 h that was significant for *ko-apx2*. After 72 h, increases in APX activity were shown in all genotypes, but it was only significant for *ko-apx2*. In Cd-exposed leaves of all genotypes, the activities of GPX, SPX and ME were increased at 24 and 72 h, however with a significantly lower activity level in *ko-apx1* compared to wildtypes. The activity of SOD was only induced in both mutants after 72 h Cd exposure. At this time point the activities of GR, ICDH and G6PDH were upregulated in all genotypes but to a significantly lower level in both mutants. GAPDH activity was only significantly increased in wildtypes and *ko-apx2*. Generally, the activities of the antioxidative enzymes were all upregulated after 72 h exposure to Cd in all genotypes, but frequently to a lower level in both mutants, and particularly in *ko-apx1* leaves.

Table 5.4. Activities of antioxidative enzymes in roots and leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 or 72 h. In the roots, the enzyme activities were expressed as mU g^{-1} FW, except for APx and SOD that were expressed as U g^{-1} FW; in the leaves, the enzyme activities were expressed as mU g^{-1} FW, except for APx that was expressed as U g^{-1} FW. Values are mean ± SE of at least 4 biological replicates. Significant differences within one time point are indicated with highlighted text p<0.05 induction and repression for an effect of treatment and significant differences for a genotype effect are shown with different letters (p<0.05).

ROOT		wildtype				*ko-apx1*				*ko-apx2*			
		control		5 µM Cd		control		5 µM Cd		control		5 µM Cd	
APX	24h	13.16 ± 1.23	a	14.36 ± 1.65	a	5.85 ± 0.45	b	6.32 ± 0.68	b	15.24 ± 1.31	a	16.57 ± 1.11	a
	72h	10.79 ± 0.72	a'	19.02 ± 1.46	a'	5.13 ± 0.40	b'	7.52 ± 0.52	b'	10.31 ± 1.46	a'	19.06 ± 1.67	a'
SOD	24h	195.64 ± 8.08		210.60 ± 34.27		174.75 ± 24.22		171.40 ± 109.93		342.92 ± 151.87		176.10 ± 28.03	
	72h	121.09 ± 28.80		264.24 ± 15.90		240.06 ± 60.71		254.59 ± 64.23		120.43 ± 30.96		333.94 ± 34.68	
GPX	24h	185.83 ± 24.49		172.43 ± 26.07		296.77 ± 67.97		269.99 ± 61.61		270.95 ± 49.33		289.99 ± 73.50	
	72h	622.93 ± 109.74		711.33 ± 179.79		539.60 ± 97.55		894.35 ± 193.39		489.34 ± 134.75		595.26 ± 90.93	
SPX	24h	507.31 ± 30.50		429.93 ± 66.52		927.15 ± 194.44		583.92 ± 108.06		897.18 ± 237.37		863.97 ± 269.54	
	72h	2555.73 ± 466.87		1729.23 ± 483.59		2164.57 ± 394.63		2278.87 ± 431.72		1084.91 ± 191.58		1360.52 ± 245.11	
CAT	24h	5.23 ± 0.70		4.72 ± 0.92		5.67 ± 0.80		4.22 ± 0.73		5.03 ± 0.63		6.56 ± 1.55	
	72h	5.25 ± 1.08		5.30 ± 1.04		3.44 ± 1.02		5.85 ± 1.31		3.73 ± 0.86		5.44 ± 0.35	
GR	24h	30.09 ± 4.41	a	41.10 ± 5.17		56.39 ± 4.93	ab	61.06 ± 7.52		66.27 ± 12.09	b	71.02 ± 7.46	
	72h	107.25 ± 16.65		105.74 ± 24.28		93.39 ± 11.13		140.78 ± 11.33		74.74 ± 12.02		114.39 ± 19.93	
ME	24h	8.83 ± 3.17		14.22 ± 6.50		5.23 ± 3.28		2.42 ± 0.53		5.76 ± 2.27		3.52 ± 1.45	
	72h	5.51 ± 1.29		5.15 ± 0.41		4.40 ± 1.49		7.19 ± 2.25		1.15 ± 0.21		1.36 ± 0.02	
ICDH	24h	10.93 ± 1.44		5.85 ± 1.47		24.65 ± 4.40		9.28 ± 3.03		22.66 ± 5.61		6.84 ± 2.01	
	72h	81.89 ± 25.82		69.13 ± 20.47		66.73 ± 18.74		110.77 ± 27.59		25.12 ± 3.13		59.01 ± 11.06	
GAPDH	24h	59.73 ± 18.99		137.29 ± 9.66		40.11 ± 5.84		95.25 ± 15.99		53.99 ± 12.27		110.44 ± 16.81	
	72h	87.50 ± 5.95		110.78 ± 12.78		83.25 ± 9.36		103.40 ± 17.93		90.82 ± 22.35		87.95 ± 11.01	

LEAF		wildtype				*ko-apx1*				*ko-apx2*			
		control		5 µM Cd		control		5 µM Cd		control		5 µM Cd	
APX	24h	6.77 ± 1.22	a	8.78 ± 1.61		6.65 ± 1.40	a	3.86 ± 0.54		15.00 ± 2.40	b	7.37 ± 0.60	
	72h	9.28 ± 2.54		15.78 ± 1.81	ab'	5.20 ± 1.30		9.98 ± 0.56	a'	5.65 ± 1.36		22.03 ± 3.02	b'
SOD	24h	270.95 ± 34.12		361.97 ± 58.84		304.01 ± 56.92		270.11 ± 41.00		260.62 ± 21.62		272.50 ± 43.23	
	72h	227.00 ± 16.62		265.34 ± 32.29		175.74 ± 19.91		286.22 ± 21.54		182.31 ± 23.48		331.38 ± 31.99	
GPX	24h	8.85 ± 2.83		65.26 ± 8.77		11.65 ± 1.34		56.74 ± 14.03		5.79 ± 0.73		52.06 ± 8.09	
	72h	5.03 ± 1.37		565.39 ± 96.06	a	3.15 ± 1.42		130.84 ± 29.93	b	2.09 ± 0.79		348.61 ± 70.97	ab
SPX	24h	32.90 ± 10.64		321.55 ± 48.23		35.16 ± 6.00		243.06 ± 65.57		21.73 ± 4.94		213.15 ± 37.74	
	72h	58.32 ± 15.08		1526.40 ± 225.23	a	49.49 ± 13.60		752.79 ± 179.80	b	45.45 ± 20.67		1109.39 ± 173.45	ab
CAT	24h	13.97 ± 1.56		20.00 ± 3.64		24.44 ± 4.86		19.81 ± 2.40		17.41 ± 2.45		16.90 ± 2.15	
	72h	23.45 ± 2.43		36.24 ± 5.57		24.78 ± 2.01		28.09 ± 3.68		25.17 ± 4.20		30.49 ± 3.65	
GR	24h	1002.65 ± 13.10		1118.75 ± 70.99		904.53 ± 57.24		1103.34 ± 75.93		774.92 ± 83.15		977.16 ± 88.12	
	72h	726.47 ± 53.21		1468.15 ± 70.42	a	642.42 ± 36.43		1060.04 ± 44.17	b	587.97 ± 65.12		1149.81 ± 113.84	b
ME	24h	233.08 ± 24.63		591.28 ± 31.28		202.60 ± 16.43		509.67 ± 61.63		183.80 ± 22.14		563.97 ± 44.26	
	72h	183.55 ± 25.01		1276.82 ± 38.11	a	179.74 ± 16.77		793.26 ± 62.35	b	138.83 ± 18.66		1080.31 ± 72.84	a
ICDH	24h	561.06 ± 12.08		542.60 ± 41.61		485.83 ± 38.35		490.46 ± 53.29		412.08 ± 51.79		445.92 ± 43.83	
	72h	402.37 ± 36.41		738.02 ± 54.93	a	350.14 ± 27.51		535.55 ± 26.54	b	297.45 ± 38.05		553.68 ± 53.44	b
G6PDH	24h	88.36 ± 2.54		116.94 ± 12.05		75.90 ± 9.02		101.26 ± 15.32		67.15 ± 6.68		96.63 ± 12.85	
	72h	78.25 ± 3.42		231.61 ± 10.11	a	58.65 ± 2.56		140.62 ± 13.75	b	51.08 ± 8.44		169.37 ± 21.87	b
GAPDH	24h	475.57 ± 94.75		680.64 ± 97.57		530.90 ± 72.39		584.88 ± 94.02		478.52 ± 38.94		511.97 ± 70.82	
	72h	303.52 ± 50.66		889.34 ± 82.17	a	237.17 ± 16.24		505.85 ± 39.09	b	286.59 ± 52.10		748.10 ± 142.99	ab

5.4. Discussion

Connecting metal toxicity and cellular redox disturbances has been the subject of intensive research (Sharma and Dietz 2009). Recently, cytosolic APXs were suggested to be regulators of (a)biotic-induced oxidative stress (Caverzan *et al.* 2012). In addition, ROS signaling and cytosolic APXs have been demonstrated to be involved amongst others during heat stress (Suzuki and Mittler 2006), light stress (Pnueli *et al.* 2003, Davletova *et al.* 2005) and salt stress (Suzuki *et al.* 2013). Previous indications for a role of cytosolic APXs during Cd stress responses in *A. thaliana* (Cuypers *et al.* 2011) prompted the functional study of APX1 and APX2 in the spatio-temporal ROS signaling and downstream responses of Cd-exposed plants. Therefore 19-days-old *ko-apx1* and *ko-apx2 A. thaliana* plants (Col-0 ecotype) were exposed to 5 µM $CdSO_4$ to investigate acute (24 h) and prolonged (72 h) cellular responses. In contrast to the knockout *APX1* line in the Wassilewskija background that showed late flowering and suppressed growth (Pnueli *et al.* 2003), *ko-apx1* and *ko-apx2* mutant Col-0 plants in the study of Suzuki *et al.* (2013) and the present study did not display any visible phenotypes under control growth conditions.

Roots are in direct contact with the nutrient solution and as Cd is highly mobile, it was readily taken up by the roots and transported to the leaves (Figure 5.1A) which is in accordance with previous studies (Smeets *et al.* 2008b, Verbruggen *et al.* 2009, Remans *et al.* 2010). Rivelli *et al.* (2014) demonstrated a clear interference of Cd exposure on nutrient uptake and especially micronutrient (Fe, Zn, Cu) translocation, which was also observed for the Cu content in our study. Whereas nutrient profiles under control conditions are the same and no genotype differences in Cd accumulation could be detected, a clear *ko-apx2* – dependent increase in Zn content after 24 and 72 h was observed in roots, whereas in leaves the Fe content was strongly elevated in both mutants in contrast to the wild-type plants (Figure 5.1). This indicates that cytosolic APX isoforms are involved in signal transduction/regulation affecting nutrient uptake and transport in plants under Cd stress.

Once Cd is taken up, it is responsible for causing toxicity in plant roots, such as stunted growth and impaired root development (Suzuki 2005, Wojas *et al.* 2007, Gallego *et al.* 2012, Remans *et al.* 2012). In our results, a reduced root fresh

weight was observed in all Cd-exposed plants after 72 h (Figure 5.2A), concomitant with an increase in %DW (Figure 5.2C). This increase in %DW could have resulted from water loss or more cell wall material making the cell wall thicker and stiffer leading to inhibited cell growth (Gomes *et al.* 2011). Lignin is a component of the cell wall and it has been demonstrated that Cd induces lignin biosynthesis, possibly limiting its entry into the roots (Ederli *et al.* 2004). Increases in lignin deposition were observed after Cd exposure in roots of *Phragmites australis* (Ederli *et al.* 2004), callus tissue culture from the roots and branches of *Camellia sinensis* (Zagoskina *et al.* 2007), roots of soybean (Bhuiyan *et al.* 2007) and in Scots pine roots (Schützendübel *et al.* 2001). Extracellular peroxidases, such as SPX, are related to the lignin biosynthesis process (Cuypers *et al.* 2002), but no increased activity was observed in wildtypes nor in *ko-apx* mutants (Table 5.4). Whereas morphologically and related to growth, no differences were observed between wildtypes and *ko-apx* mutants in our experimental set-up, differences in nutrient uptake and translocation were observed. This indicates that other regulation mechanisms and/or signal transduction pathways might be activated to counteract Cd-evoked responses in plants.

Cadmium-induced oxidative stress has been observed in many plant species (Sandalio *et al.* 2001, Romero-Puertas *et al.* 2004, Smeets *et al.* 2005, Garnier *et al.* 2006) and it was demonstrated that Cd exposure enhances H_2O_2 production in *A. thaliana* (Cuypers *et al.* 2011). There are different pathways by which H_2O_2 can be perceived, e.g. via 1) oxidative signal-inducible kinase (OXI1) and 2) redox-sensitive transcription factors, such as HSF21 (Davletova *et al.* 2005). Signaling genes involved in the OXI1-MAPK cascade were examined, because they were previously found to be responsive to Cd exposure in *A. thaliana* (Opdenakker *et al.* 2012a, Smeets *et al.* 2013). Moreover they are involved in activating gene expression of ROS producing and antioxidative genes (Mittler *et al.* 2004), which are important characteristics of Cd-induced responses (Smeets *et al.* 2008b, Cuypers *et al.* 2011, Opdenakker *et al.* 2012a). No induction of *OXI1* was observed in the roots of *ko-apx1* (Table 5.2), which indicates that this genotype is less susceptible to Cd-induced H_2O_2 accumulation or that less H_2O_2 is present compared to the other genotypes. After prolonged exposure to Cd, the gene expression of downstream MAPKinases and

transcription factors *HSF21* and *ZAT12* was also significantly lower or even downregulated in *ko-apx1* plants, meaning that APX1 is part of the Cd-induced signal transduction in roots of *A. thaliana*. Rizhsky *et al.* (2004) suggested that ZAT12 is a key component of the signaling network activated by oxidative stress in *A. thaliana*, thereby controlling the expression of other transcription factors, such as *ZAT7* that was also suppressed in *ko-apx1*. Davletova *et al.* (2005) proposed a sequential scheme for a role of APX1 in moderate light stress in which H_2O_2 is sensed by *HSF21* - *ZAT12* - *APX1*, which in turn controls the transcript level of *RBOHD*, essential for amplification of the ROS signal and hence defense response. This is in accordance with the observations in our study, indicating that *ko-apx1* mutants showed a disturbed signaling pathway, and hence a disturbed activation of antioxidative genes and activities. Indeed *ko-apx1* mutants showed a constitutive higher *CAT* expression (Figure 5.4), but lower APX activity in comparison with the other genotypes (Table 5.4). Such a compensation mechanism was also seen in transgenic tobacco plants expressing antisense RNA for APX1, which have a higher CAT activity compared to their wildtypes (Rizhsky *et al.* 2002). However, in our study no increase in CAT activity was observed in *ko-apx1* roots. Increased activities in APX or SOD upon Cd exposure were only observed in wildtypes and *ko-apx2* mutants, however the underlying transcriptional regulation of different SOD isoforms via the transcription factor SPL7 – miRNA398 – CSD or SPL7 – FSD (Sunkar *et al.* 2006, Sunkar *et al.* 2007, Yamasaki *et al.* 2009) was the same in all genotypes.
In general, whereas no differences in Cd-induced root growth responses were observed, the micronutrient profile is strongly affected in *ko-apx2* mutants, whereas a suppressed H_2O_2 sensing and signaling via HSF21 and OXI1 is observed in *ko-apx1* mutants.

Cadmium exposure did not affect leaf biomass in our experimental set-up, however the %DW increased in Cd-exposed plants that might be linked to lignification (Figure 5.2D). In all Cd-exposed plants, GPX and SPX activities rose (Table 5.4), which might be beneficial to capture Cd ions in the cell wall and within our time frame without negative effects on growth. It should be noted that the activities of both enzymes are lower in *ko-apx1* mutants after Cd exposure. This could be due to alterations in root-shoot signaling as a result of

suppressed root signal transduction (cfr. supra), which in its turn might affect leaf responses. Whether this mitigates long-term Cd tolerance or sensitivity needs to be further explored.

Taking a closer look on Cd-induced H_2O_2 accumulation, this was less pronounced in *ko-apx1* mutants compared to wild-type and *ko-apx2* plants. Based on a suppressed signal transduction in roots, it is suggested that *ko-apx1* plants experience a diminished accumulation of H_2O_2 in leaves (Figure 5.3). Despite the lower amount of H_2O_2 signaling molecules, a more pronounced increase in ZAT transcription factors, as compared to wildtypes, was observed according to Davletova *et al.* (2005), in response to moderate light stress, indicating that APX1 definitely is involved in controlling signaling pathways. Also a more pronounced increase in ethylene biosynthesis genes was noticed in *ko-apx1* as compared to wild-type plants pointing towards ethylene as a signaling molecule in leaves after Cd exposure. Schellingen *et al.* (2014) have shown that both biosynthesis genes are crucial in the ethylene production in the early response to Cd stress. Ethylene, in turn, activates gene expression of signaling and antioxidative genes. Recently, it has been shown that ethylene signaling activates the MKK9-MPK3/6 module (Hahn and Harter 2009), which could be an alternative signal transduction pathway in *ko-apx1* mutants upon Cd stress. At metabolic level GSH biosynthesis, important as Cd chelator and antioxidant (Jozefczak *et al.* 2012), was stimulated in Cd-exposed *ko-apx1* mutants, although to a lower extent in comparison to both other genotypes. Whereas all Cd-exposed plants in the present study seemed to be able to cope with Cd stress and adopt a new metabolic equilibrium, *ko-apx2* mutants seemed to rely on the AsA-GSH cycle to tackle Cd-evoked ROS production. Besides GSH biosynthesis, only *ko-apx2* mutants showed increases in AsA content and APX activity (Table 5.1 and 5.4). It is known that Cd influences the redox cycle in plant cells using reducing power which is clearly stimulated in all Cd-exposed genotypes, according to the studies of Smeets *et al.* (2005) and Semane *et al.* (2007).

Whereas no clear differentiation in gene expression between wildtypes and *ko-apx2* plants was observed, a strong elevation in Fe content and SOD activity in both mutant genotypes was observed in contrast to the wild-type plants. FeSOD is located in the chloroplast, requires Fe as a cofactor and its activity is

enhanced under Cd exposure (Jozefczak *et al.* 2014). Transgenic maize lines overproducing an *A. thaliana* FeSOD in the chloroplasts suffered less from paraquat-induced damage as indicated by higher photosynthetic activity (Vanbreusegem and Inzé 2002). In addition, Fe deficiency leads to a decrease in SOD activity in *Brassica juncea* exposed to Cd, while increases in SOD activity were found in the presence of Fe providing Cd stress tolerance in these plants (Muneer *et al.* 2011). Furthermore, it is shown in *Brassica juncea* that Fe has a role in stabilizing thylakoid multiprotein complexes and hence provided stability to the chloroplast under Cd stress (Qureshi *et al.* 2010). Thus, the increased Fe accumulation and SOD activity in both mutants suggested a protection for the chloroplasts. Because *ko-apx* mutants have a loss in APX1 or APX2, these mutants are more sensitive to light, therefore they need a bypass to counteract this light sensitivity especially under Cd stress because Cd also exerts an effect on photosynthesis.

In conclusion, our results indicate that APX1 and APX2 are involved in the spatio-temporal ROS signaling and downstream responses of Cd-exposed plants (Figure 5.5). An important role for APX1 in the cellular signaling during Cd-induced toxicity in roots of *A. thaliana* is observed because plants lacking APX1 show a suppressed expression of genes involved in signal transduction leading to a disturbed activation of the antioxidative system. Probably an altered root-to-shoot signaling affects leaf responses in *ko-apx1*, *i.e.* lower accumulation of H_2O_2 and increased ethylene biosynthesis leading to altered gene expression and activities. On the other hand, APX2 has a role in the nutrient profile as well as in the metabolism of AsA, which are both strongly affected in *ko-apx2* after Cd exposure. Finally, it is considered that the stimulation of Fe accumulation and SOD activity in both *ko-apx* mutants is needed for the protection of the chloroplasts under Cd stress.

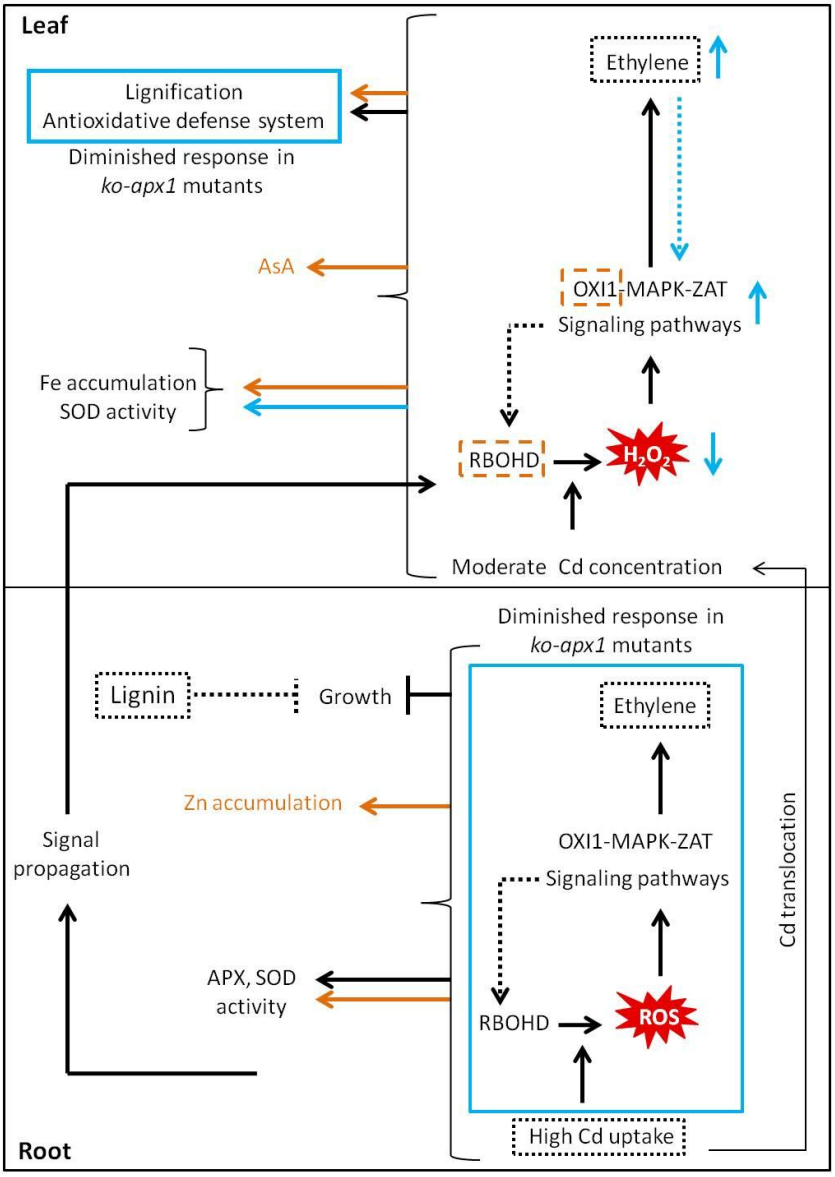

Figure 5.5. Overview of oxidative signaling pathways and defense responses in Cd-exposed *Arabidopsis thaliana* roots and leaves. Black lines represent responses in wildtypes, while different regulated responses in the mutants are represented by blue lines (*ko-apx1*) and orange lines (*ko-apx2*). Dashed lines represent delayed responses. Dotted lines represent pathways found in literature; Cd induces ethylene production in roots and leaves and stimulates synthesis of lignin, which in turn inhibits growth.

CHAPTER 6

Ascorbate and the activity of GMP interfere with oxidative signaling and mediates Cd-induced oxidative stress in *Arabidopsis thaliana*

An Bielen, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2014). Ascorbate and the activity of GMP interfere with oxidative signaling and mediates Cd-induced oxidative stress in *Arabidopsis thaliana*. In preparation.

Abstract

Ascorbate (AsA) is an important component of the plant's antioxidative system that is affected after cadmium (Cd) and copper (Cu) exposure in *Arabidopsis thaliana* plants. Amongst others, AsA is involved in the reduction of hydrogen peroxide (H_2O_2) via ascorbate peroxidase in the ascorbate–glutathione cycle. To elucidate the role of AsA and GDP-D-mannose pyrophosphorylase (GMP; involved in AsA synthesis and cell wall processes) in Cd- and Cu-induced oxidative signaling and stress responses, AsA deficient mutants, *vtc2* and *vtc1-1* respectively, were grown on hydroponics, exposed to 5 µM Cd or 2 µM Cu during 24 h and investigated at different biological levels. The *vtc1-1* mutants were more Cd sensitive in comparison to wildtypes and *vtc2* mutants as was observed from increased lipid peroxidation and decreased fresh weights. In addition low H_2O_2 levels were observed that in turn resulted in a lack of oxidative signaling and a lack of changes in antioxidative defense responses. These results demonstrate that AsA itself as well as the activity of GMP are important in provoking a proper signal transduction and hence play a role in Cd-induced stress responses.

Chapter 6

6.1. Introduction

Due to industrial activities (e.g. metal ore mining and processing) and agricultural practices (e.g. use of Cd containing P-fertilizer and Cu containing pesticides), an increased amount of toxic metals has been released into the environment. These metals can accumulate in crops grown on metal-contaminated soils leading to decreased crop production and quality (Jarüp 2003, Islam *et al.* 2007, Cockell *et al.* 2008). It has been demonstrated that accumulation of Cd or Cu in *Arabidopsis thaliana* plants can cause adverse effects (Cuypers *et al.* 2011). Although Cu is an essential element required for growth and development, at higher concentrations it can become toxic leading to chlorosis, necrosis, reduced biomass and inhibition of shoot and root growth (Yruela 2005, Yruela 2009). Moreover, the redox properties that make Cu an essential element, also contribute its inherent toxicity. In contrast, Cd is a non-redoxactive, non-essential element that negatively affects plant growth and development (Gallego *et al.* 2012). It is known that both Cd and Cu can induce oxidative stress as they can cause an imbalance between the rate of reactive oxygen species (ROS) production and their degradation (Smeets *et al.* 2009, Cuypers *et al.* 2011). The oxidative burst is a common response of plant cells to environmental fluctuations, leading to an augmentation of ROS, such as superoxide ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2), which have an oxidizing function that can be harmful for the plant cell, but are also involved in stress signaling. Also after Cd or Cu exposure the H_2O_2 that is immediately produced might be a key molecule that can trigger signal transduction events after plant metal exposure (Mithöfer *et al.* 2004, Smeets *et al.* 2009). To protect plant cells from oxidative damage while still allowing ROS signaling, plants have evolved an antioxidative defense system consisting of enzymes and metabolites. Ascorbate (AsA) is a well-known and important metabolite of the plant's antioxidative system. As primary antioxidant it can reduce ROS directly, or indirectly via ascorbate peroxidase (APX) activity in the ascorbate-glutathione (AsA-GSH) cycle (Figure 1.3). In this cycle, enzymes and metabolites act together to detoxify H_2O_2 by the reducing power derived from NADPH (Foyer and Noctor 2005b, Halliwell 2006).

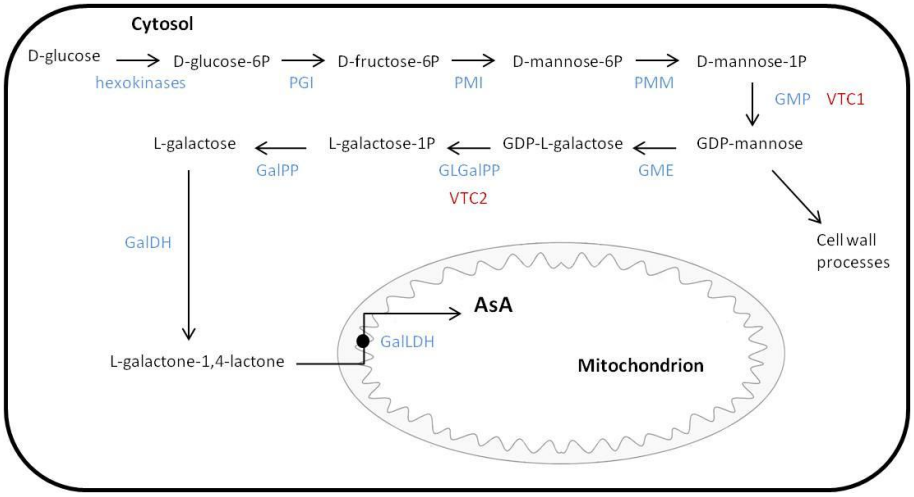

Figure 6.1. Representation of the biosynthesis of AsA. The biosynthesis of AsA takes place in the cytosol, except the last step occurs in the mitochondrion. Abbrevations: PGI, phosphoglucose isomerase; PMI, phosphomannose isomeras; PMM, phosphomannomutase; GMP, GDP-mannose-pyrophosphorylase; GME, GDP-mannose-3',5'-epimerase; GLGalPP, GDP-L-galactose phosphorylase; GalPP, L-galactose-1-P-phosphatase; GalDH, L-galactose dehydrogenase; GalLDH, L-galactone-1,4-lactone dehydrogenase; AsA, ascorbate. VTC1 and VTC2 are alternative names for the genes encoding the respective enzymes as these genes were identified from vitamin C deficient mutants.

In plants, AsA is an abundant water-soluble metabolite with essential roles in multiple normal physiological and developmental processes, and in the regulation of defense and survival of plants under stress conditions (reviewed in Bielen *et al.* 2013). It has been shown that metal stress can disturb AsA levels and functions. There was an indication that AsA has a role in the acclimation of plants to metal stress, because an altered AsA pool was observed in various plant species during Cd stress (Schützendübel *et al.* 2001, Aravind and Prasad 2005, Smeets *et al.* 2005, Cuypers *et al.* 2011, Mohamed *et al.* 2012) and Cu stress (Gupta *et al.* 1999, Cuypers *et al.* 2000, Drazkiewicz *et al.* 2003, Tewari *et al.* 2006, Smeets *et al.* 2009, Cuypers *et al.* 2011, Thounaojam *et al.* 2012). Ascorbate occurs as a redox couple and can be involved in tuning cellular signaling pathways under (a)biotic stress conditions (Pignocchi and Foyer 2003b,

Foyer and Noctor 2005b), but little is known on its role under Cd or Cu exposure. To gain more insight into the importance of AsA in oxidative stress signal transduction during Cd or Cu stress, oxidative stress related responses on the molecular level were examined after 24 h exposure in wild-type and AsA deficient (*vtc1-1* mutant) *A. thaliana* plants. The results using this mutant indicated that AsA and its redox state play an important regulatory role for the downstream responses under Cd stress. The *vtc1-1* mutant has a defect in the GDP-D-mannose pyrophosphorylase (GMP) enzyme found in the initial part of the biosynthesis pathway of AsA (Figure 6.1), resulting in a low AsA level (<30%). Since the GMP-derived GDP-sugar intermediates that are formed by the GMP enzyme are also involved in the synthesis of cell wall polysaccharides and glycoproteins (Smirnoff 2000b), the study was continued to investigate whether the reduced level of AsA itself, or the disturbed cell wall processes are responsible for the modified Cd-induced stress responses in the *vtc1-1* mutant. To this purpose, a second AsA deficient mutant was used in which GMP-derived GDP-sugar intermediates are not limiting. The *vtc2* mutant has low AsA levels due to a defect in the GDP-L-galactose phosphorylase enzyme (GLGalPP) (Figure 6.1), which occurs later in the pathway after the formation of GMP-derived GDP-sugar intermediates. Downstream responses of wild-type, *vtc1-1* and *vtc2* *A. thaliana* plants were compared after exposure to Cd for 24 h to reveal the role of AsA in oxidative signaling during Cd stress.

6.2. Experimental design and methodology

To investigate the sensitivity of the mutation, *A. thaliana* plants (wildtypes and *vtc1-1*) were grown on vertical agar plates to analyze the root growth. After 7 days of growth on control plates, a homogenous subset of the seedlings was transferred to treatment plates covering a concentration range of Cd (0-10 µM $CdSO_4$) and cultured for another 7 days. In order to explore the reduced AsA levels during metal exposure, plants were grown on hydroponics as described previously (Chapter 3, section 3.2). After three weeks the plants were exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h. Besides biomass and metal content, indicators of oxidative stress were analyzed. At the transcriptional level, gene expression was analyzed for 1) oxidative stress related genes and 2) genes

Ascorbate and the activity of GMP interfere with Cd-induced oxidative signaling involved in signal transduction At metabolic level, lipid peroxidation was investigated.

To unravel the interaction of AsA and the GMP activity in Cd-induced oxidative signaling, *A. thaliana* plants (wildtypes, *vtc1-1* and *vtc2*) were grown on hydroponics. After three weeks the plants were exposed to 5 µM $CdSO_4$ for 24 h. Besides biomass and metal content, indicators of oxidative stress were monitored at different biological levels. At the transcriptional level, gene expression was analyzed for 1) oxidative stress related genes, 2) genes involved in signal transduction and 3) genes related to ethylene biosynthesis/signaling. At metabolic level, H_2O_2 content, lipid peroxidation, antioxidative enzyme activities and concentration of antioxidative metabolites (AsA and GSH) were investigated.

6.3. Results

Response of *vtc1-1* mutants to Cd or Cu exposure

6.3.1. Metal uptake

Elemental profiles were determined in 19-days-old wild-type and *vtc1-1* *A. thaliana* plants exposed for 24 h to 5 µM $CdSO_4$ or 2 µM $CuSO_4$. Cadmium contents were significantly increased in both roots and leaves of all genotypes exposed to Cd and no genotype effects were detected (Figure 6.2A). When plants were exposed to 2 µM Cu, Cu ions were taken up in excess by the roots and were mainly retained in roots as the concentration of Cu in leaves increased but remained relatively low (Figure 6.2B). Also for Cu accumulation, no genotype differences were observed.

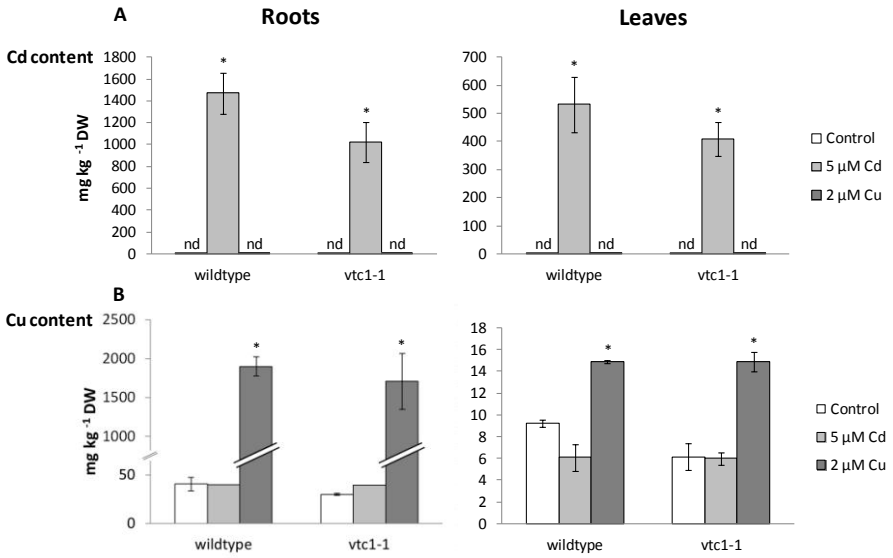

Figure 6.2. Cd content (A) and Cu content (B) are expressed as mg kg^{-1} DW in roots and leaves of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h or grown under control conditions. Values are mean ± SE of three biological replicates. Significant differences relative to the unexposed genotype are shown: *p<0.05. Abbreviation nd, not detected.

6.3.2. Growth responses

In hydroponic culture, under normal circumstances, an altered leaf morphology was found for *vtc1-1*, showing more elongated leaves as compared to the more round-shaped wild-type leaves (Figure 6.3A,B). Also under normal conditions, a lower root fresh weight was observed in *vtc1-1* roots compared to wildtypes (Figure 6.4A).

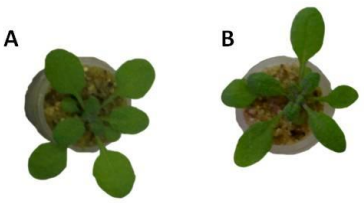

Figure 6.3. Morphology between wildtypes (A) and *vtc1-1* mutants (B). Pictures were taken at day 18 (one day before exposure).

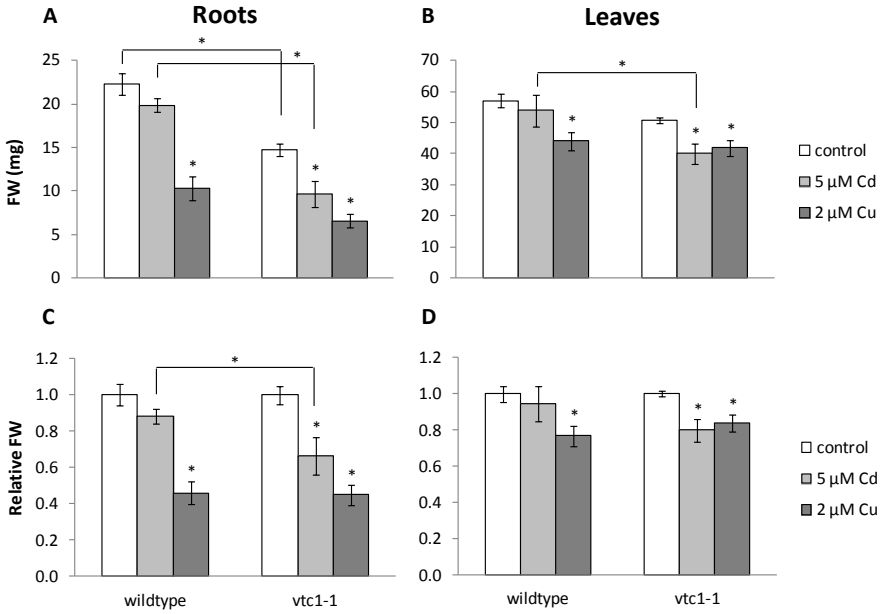

Figure 6.4. Absolute fresh weight (FW, mg) (A,B) and relative FW (C,D) in roots and leaves of 19-days-old *Arabidopsis thaliana* exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h or grown under control conditions. Values are mean ± SE of ten biological replicates. Significant differences relative to the unexposed genotype are shown: *p<0.05. Significant genotype differences are indicated with brackets.

After Cd exposure, no significant effects on root or leaf fresh weight were noticed in wild-type plants, whereas *vtc1-1* mutants showed significant decreases (Figure 6.4A,B). Moreover, relative root fresh weight observed after Cd exposure was significantly lower in *vtc1-1* compared to wildtypes (Figure 6.4C). When plants were exposed to Cu, decreased root and leaf fresh weights were found for both genotypes. In roots, a decreased fresh weight was concomitant with an increased dry weight percentage (%DW) (Figure 6.5A). The roots of the wildtypes did not show an increased %DW after Cd exposure, while the *vtc1-1* roots did, which is in accordance with the fresh weight data.

In leaves, an increase in %DW was found only in Cu-exposed wild-type plants and not in the *vtc1-1* mutant. Additionally, under control conditions, a significantly higher %DW was observed in *vtc1-1* leaves compared to wildtypes. The obtained results indicated that both genotypes showed nearly the same

sensitivity to Cu exposure, while *vtc1-1* mutants were more sensitive to Cd stress as compared to wildtypes.

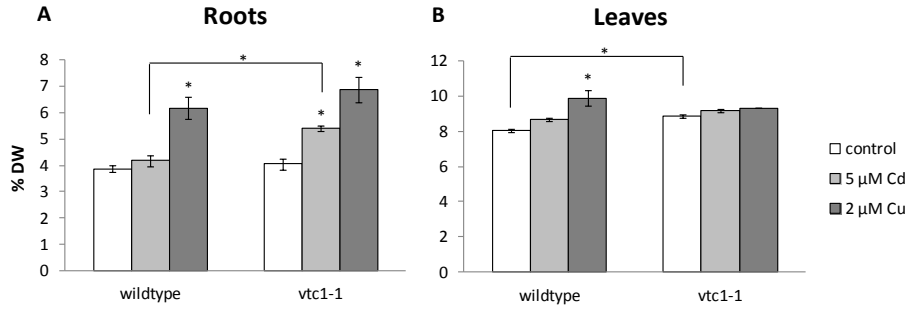

Figure 6.5. Dry weight percentage (%DW) was measured in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h or grown under control conditions. Values are mean ± SE of three biological replicates. Significant differences relative to the unexposed genotype are shown: *p<0.05. Significant genotype differences are indicated with brackets.

Root development during Cd and Cu exposure was investigated with more detail in *A. thaliana* wildtypes and *vtc1-1* mutants using a vertical agar plate growth system. Plants were germinated on control medium before being transferred after 7 days to medium containing a range of increased Cd or Cu concentrations. At the time of transfer, primary root length was significantly smaller in the *vtc1-1* mutants compared to wild-type plants (Figure 6.6A). This was similar to the observation in hydroponics and may indicate that *vtc1-1* mutant roots grow slower, however, the *vtc1-1* mutants had also a longer average lateral root length than wild-type plants when grown under control conditions (Figure 6.6B). Since there is a difference in root growth between the genotypes under control conditions, root growth data were normalized to the control condition within the genotype (Figure 6.6C,E). The most obvious differences were seen in lateral root emergence (number of laterals), which was more sensitive to the higher Cd concentrations in the *vtc1-1* mutant, and mean lateral root length, which was more sensitive to higher Cu concentrations (Figure 6.6C,E).

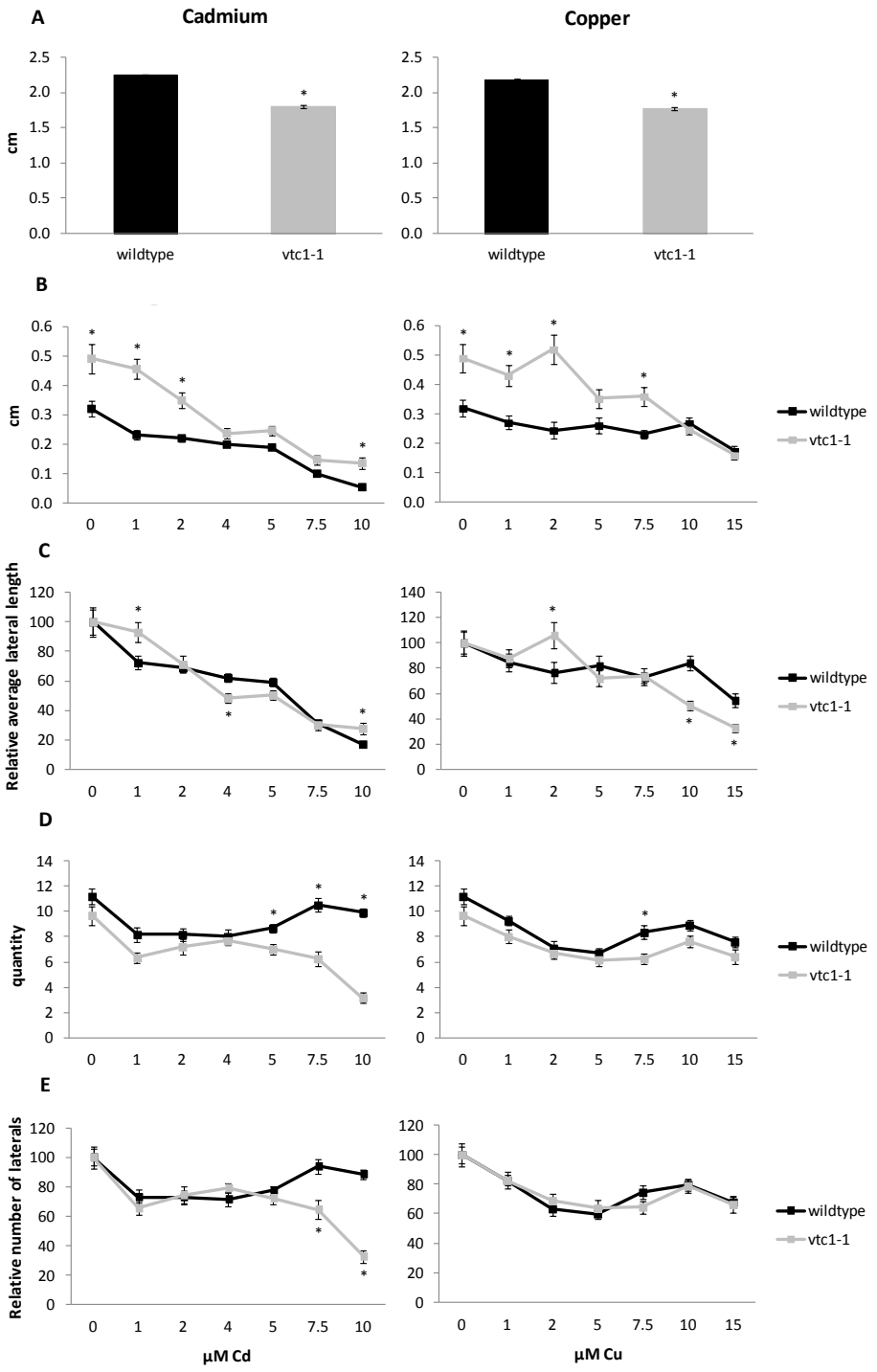
A
Cadmium
Copper
cm
wildtype
vtc1-1
B
C
Relative average lateral length
D
quantity
E
Relative number of laterals
µM Cd
µM Cu

Figure 6.6. Root development parameters in *Arabidopsis thaliana* wild-type and *vtc1-1* mutant seedlings. Seedlings were grown for 7 days in vertical agar plates before transfer to treatment plates containing $CdSO_4$ (0-10 µM Cd, on the left) or $CuSO_4$ (0-15 µM Cu, on the right). (A) Primary root length of 7d old plants (cm) before transfer (for the plants transferred to the respective Cd or Cu concentrations), (B) mean lateral root length (cm), (C) relative average lateral root length within the genotypes, (D) number of lateral roots and (E) relative number of lateral roots within the genotypes after 7 days exposure. Significant difference between genotypes within a treatment are indicated: *p<0.05.

6.3.3. Lipid peroxidation in A. thaliana wild-type plants is different from vtc1-1 mutants

The plasma membranes are considered as a primary target for metal-induced damage. The oxidation of membrane lipids was investigated by determining tissue levels of TBA reactive metabolites (TBArm). After Cd exposure, increased TBArm levels were only found in roots of *vtc1-1* plants, while Cu exposure induced lipid peroxidation in roots and leaves of both genotypes. Moreover, TBArm levels in Cu-exposed *vtc1-1* leaves were significantly lower than those observed in wildtypes (Figure 6.7A,B).

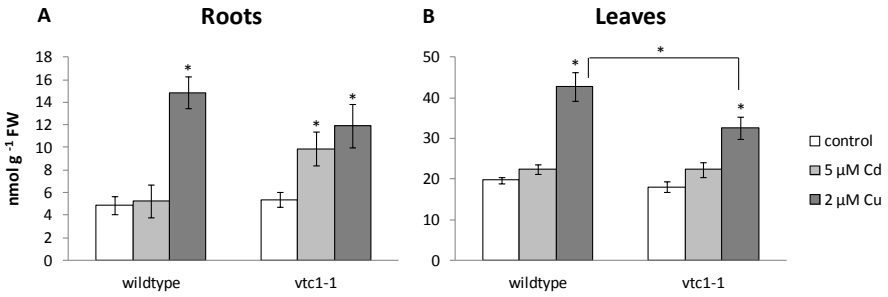

Figure 6.7. TBA reactive metabolites are expressed as nmol g^{-1} FW in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h or grown under control conditions. Values are mean ± SE of six biological replicates. Significant differences relative to the unexposed genotype are shown: *p<0.05. Significant genotype differences are indicated with brackets.

6.3.4. Alterations in the pro- and antioxidant gene network in A. thaliana during Cu versus Cd stress

6.3.4.1. Pro-oxidative genes

In wild-type roots, significantly increased transcript levels of *RBOHD* (respiratory burst oxidase homologue) and *LOX1* (lipoxygenase) were observed after Cd exposure, while no inductions or significantly lower levels were noticed in *vtc1-1* roots (Table 6.1). Under Cu exposure, the expression level of *RBOHD* and *LOX1* was enhanced in both genotypes. *RBOHC* and *LOX2* expression was unaltered after Cd exposure, but decreased and increased, respectively, after Cu exposure in both genotypes (Table 6.1).

In leaves exposed to Cd, significantly induced *RBOHC*, *RBOHD*, *LOX1* and *LOX2* expression levels were found in wild-type plants, while these inductions were significantly lower or even absent in *vtc1-1* leaves (Table 6.2). After Cu exposure, no significant changes were observed in the expression level of ROS-producing genes, except for *LOX2* transcript levels that were increased in both genotype leaves. In general, the difference between wildtype and *vtc1-1* mutant was more pronounced in plants exposed to Cd.

6.3.4.2. Antioxidative gene expression

The antioxidative genes measured included different isoforms of superoxide dismutase (*SOD*), catalase (*CAT*) and *APX*, as well as different isoforms of genes involved in the AsA-GSH cycle, *i.e.* dehydroascorbate dehydrogenase (*DHAR*), monodehydroascorbate reductase (*MDHAR*) and glutathione reductase (*GR*). The results normalized to the control condition within a genotype are presented in Table 6.1 (roots) and 6.2 (leaves). This analysis highlights differences in induction or repression between genotypes, but does not take into account any genotype differences that exist under control conditions. Therefore, significant differences that were observed between the genotypes under control conditions in the respective experiments are summarized in Table 6.3. For example, in *vtc1-1* mutants, *FSD1* expression was significantly higher in the roots as compared with wildtypes under control conditions (Table 6.3A).

Table 6.1. Transcript levels in roots of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h. Fold changes in expression levels within a genotype are relative to the non-exposed plants (0 µM) (1.00 ± SE). Significant treatment effects within a genotype are highlighted: p<0.05 induction, repression; p<0.01 induction, repression; p<0.001 induction, repression. Genotype*treatment interaction effects are indicated in the last column of each treatment, and with bold font. The significant differences in fold changes between the two treatments within one genotype are indicated with: *p<0.05. Values are mean ± SE of four biological replicates.

ROOT	control		5 µM Cd			2 µM Cu			Differences fold changes Cd-Cu	
	wildtype	*vtc1-1*	wildtype	*vtc1-1*	Interaction	wildtype	*vtc1-1*	Interaction	wildtype	*vtc1-1*
$O_2^{\cdot-}$ scavenging										
MSD1	1.00 ± 0.05	1.00 ± 0.04	1.21 ± 0.05	1.12 ± 0.04		**1.79 ± 0.20**	**1.13 ± 0.07**	**p<0.01**	*	
CSD1	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.04	0.73 ± 0.06		3.04 ± 0.57	2.04 ± 0.19		*	*
CSD2	1.00 ± 0.10	1.00 ± 0.07	0.90 ± 0.08	0.77 ± 0.05		**2.85 ± 0.68**	**1.27 ± 0.11**	**p<0.05**	*	
FSD1	1.00 ± 0.17	1.00 ± 0.15	**155.46 ± 60.78**	**24.57 ± 4.59**	**p<0.01**	1.27 ± 0.56	0.27 ± 0.06		*	*
H_2O_2 scavenging										
CAT1	1.00 ± 0.07	1.00 ± 0.03	**2.55 ± 0.35**	**0.93 ± 0.04**	**p<0.05**	9.01 ± 0.28	2.16 ± 0.19		*	*
CAT2	1.00 ± 0.02	1.00 ± 0.04	**0.98 ± 0.05**	**0.64 ± 0.02**	**p<0.001**	0.42 ± 0.02	0.51 ± 0.05		*	
CAT3	1.00 ± 0.05	1.00 ± 0.03	**1.24 ± 0.13**	**0.85 ± 0.05**	**p<0.05**	1.53 ± 0.17	1.68 ± 0.25			*
APX1	1.00 ± 0.09	1.00 ± 0.08	0.89 ± 0.04	0.90 ± 0.05		**1.53 ± 0.14**	**1.03 ± 0.14**	**p<0.05**	*	
APX2	1.00 ± 0.16	1.00 ± 0.15	3.13 ± 0.88	3.53 ± 0.89		3.53 ± 0.37	3.24 ± 0.66			
APX3	1.00 ± 0.01	1.00 ± 0.02	1.19 ± 0.03	1.08 ± 0.01		1.35 ± 0.01	1.39 ± 0.11		*	*
APX4	1.00 ± 0.07	1.00 ± 0.14	0.71 ± 0.15	0.78 ± 0.14		0.29 ± 0.05	0.33 ± 0.09		*	*
APX5	1.00 ± 0.03	1.00 ± 0.04	0.66 ± 0.00	0.81 ± 0.02		1.03 ± 0.05	1.14 ± 0.19		*	*
APX6	1.00 ± 0.01	1.00 ± 0.02	1.69 ± 0.28	1.42 ± 0.02		1.84 ± 0.03	2.21 ± 0.34			
APX7	1.00 ± 0.03	1.00 ± 0.05	**0.75 ± 0.05**	**1.11 ± 0.05**	**p<0.001**	1.54 ± 0.08	1.45 ± 0.07		*	*
sAPX	1.00 ± 0.03	1.00 ± 0.03	1.03 ± 0.07	1.08 ± 0.08		1.45 ± 0.01	1.14 ± 0.15		*	
tAPX	1.00 ± 0.09	1.00 ± 0.09	0.61 ± 0.05	0.66 ± 0.04		0.15 ± 0.02	0.15 ± 0.02		*	*
MDHAR1	1.00 ± 0.02	1.00 ± 0.11	1.53 ± 0.15	1.27 ± 0.06		3.23 ± 0.21	3.83 ± 0.47		*	*
MDHAR2	1.00 ± 0.03	1.00 ± 0.03	1.51 ± 0.11	1.27 ± 0.03		1.21 ± 0.09	1.31 ± 0.17			
MDHAR3	1.00 ± 0.03	1.00 ± 0.07	1.43 ± 0.16	1.40 ± 0.06		0.82 ± 0.15	0.87 ± 0.17		*	*
MDHAR4	1.00 ± 0.07	1.00 ± 0.02	1.35 ± 0.08	1.16 ± 0.02		1.25 ± 0.13	1.57 ± 0.06			*
MDHAR5	1.00 ± 0.06	1.00 ± 0.06	0.61 ± 0.06	0.75 ± 0.06		0.47 ± 0.07	0.36 ± 0.01			*
DHAR1	1.00 ± 0.09	1.00 ± 0.06	1.23 ± 0.07	0.99 ± 0.11		**2.31 ± 0.50**	**1.08 ± 0.10**	**p<0.05**		
DHAR2	1.00 ± 0.07	1.00 ± 0.04	1.29 ± 0.12	1.26 ± 0.06		1.59 ± 0.57	1.78 ± 0.13		*	
DHAR3	1.00 ± 0.07	1.00 ± 0.08	0.62 ± 0.04	0.94 ± 0.14		1.25 ± 0.24	1.65 ± 0.11		*	*
GR1	1.00 ± 0.05	1.00 ± 0.11	1.54 ± 0.05	1.54 ± 0.06		1.66 ± 0.07	1.51 ± 0.05			
GR2	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.07	0.85 ± 0.01		0.61 ± 0.06	0.81 ± 0.06		*	
ROS production										
RBOHC	1.00 ± 0.07	1.00 ± 0.05	1.15 ± 0.13	1.09 ± 0.02		0.37 ± 0.05	0.30 ± 0.01		*	*
RBOHD	1.00 ± 0.16	1.00 ± 0.19	2.89 ± 0.00	1.73 ± 0.14		3.40 ± 0.38	3.38 ± 0.53			*
LOX1	1.00 ± 0.06	1.00 ± 0.11	**5.05 ± 0.86**	**2.26 ± 0.08**	**p<0.01**	28.51 ± 0.82	32.89 ± 5.62		*	*
LOX2	1.00 ± 0.84	1.00 ± 0.63	4.37 ± 1.31	0.82 ± 0.12		8.19 ± 2.45	12.46 ± 4.37			*
Signaling										
OXI1	1.00 ± 0.07	1.00 ± 0.06	3.01 ± 0.17	2.29 ± 0.19		4.65 ± 1.25	4.19 ± 0.95			*
MPK3	1.00 ± 0.03	1.00 ± 0.13	**3.15 ± 0.01**	**1.39 ± 0.08**	**p<0.01**	1.73 ± 0.42	1.38 ± 0.22		*	
MPK6	1.00 ± 0.02	1.00 ± 0.07	1.30 ± 0.07	1.18 ± 0.06		0.69 ± 0.02	0.99 ± 0.17		*	
WRKY25	1.00 ± 0.04	1.00 ± 0.11	**2.86 ± 0.38**	**1.60 ± 0.14**	**p<0.01**	**3.66 ± 0.36**	**2.01 ± 0.17**	**p<0.01**		
ZAT12	1.00 ± 0.22	1.00 ± 0.13	5.47 ± 0.60	2.89 ± 0.53		36.97 ± 7.38	20.53 ± 3.21		*	*
HSF21	1.00 ± 0.11	1.00 ± 0.16	3.09 ± 0.21	2.23 ± 0.21		1.98 ± 0.39	1.88 ± 0.37			

Ascorbate and the activity of GMP interfere with Cd-induced oxidative signaling

While the induction of *FSD1* by Cd was lower in the *vtc1-1* mutant (Table 6.1), the transcript levels after Cd exposure may still be higher in this mutant due to the further upregulation of already high transcript levels. Where no such genotype differences exist, differential responses of the genotypes to metal exposure is evident from the genotype*treatment interaction analysis in Table 6.1 and 6.2.

Regarding root responses (Table 6.1), when plants were exposed to Cd, the transcript levels of *CAT1*, *CAT2* and *CAT3* were significantly lower in *vtc1-1* compared to wildtypes, while the expression of *APX7* was significantly higher. When plants were exposed to Cu, the expression of *MSD1*, *CSD2*, *APX1* and *DHAR1* were significantly lower in *vtc1-1* compared to wild-type roots. Moreover, after Cd exposure the expression of antioxidative genes generally remained unaltered in *vtc1-1* mutants, while somewhat more changes in expression were observed in wildtypes.

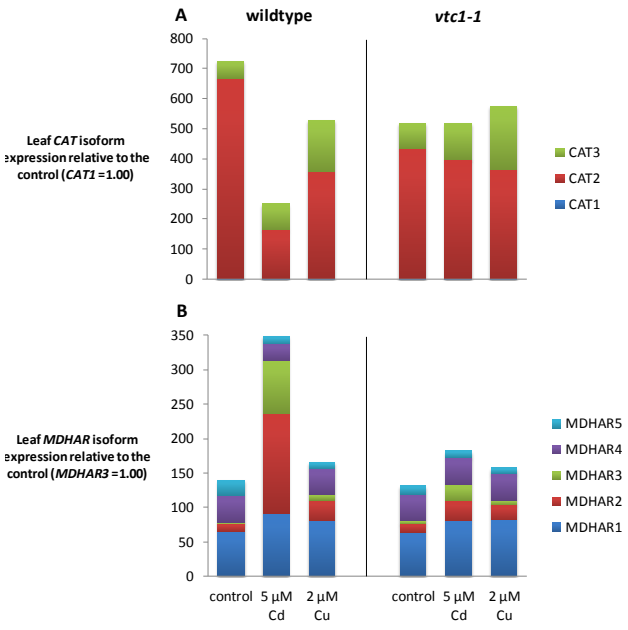

Figure 6.8. Exposure to Cd has an effect on the relative transcript abundance of the *CAT* (A) and *MDHAR* (B) gene family members in the leaves of *Arabidopsis thaliana* plants. Plants were exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h. Data are given as the mean abundance in 4 biological independent replicates relative to the control (24 h, 0 µM) with the abundance of the lowest expressed gene family member set at 1.00.

In leaves under control conditions, genotype differences were observed showing a significantly downregulated expression of *CAT2* and *MDHAR5* and an upregulated expression of *MDHAR3* in *vtc1-1* as compared to wildtypes (Table 6.3). In wild-type plants exposed to Cd, most genes involved in AsA-GSH cycle showed significant induction or repression, while this was not the case in *vtc1-1* plants that showed no altered expression level or an increase or decrease that was significantly less pronounced. A similar observation was made for CAT gene expression. Analysis of the gene families demonstrated that wild-type plants, as opposed to *vtc1-1* mutants, showed strong variations in *CAT* and *MDHAR* expression when plants were exposed to Cd (Figure 6.8A,B). Indeed, the decrease in *CAT2* expression in wild-type plants is not compensated by higher expression of other isoforms, such that the total level of CAT expression is decreased. For MDHAR the increase in expression in wild-type plants under Cd exposure is mostly attributable to *MDHAR2* upregulation, and somewhat less to *MDHAR3* upregulation, while this is much less pronounced in the *vtc1-1* mutants. After Cu exposure no such differences between the genotypes are apparent.

Table 6.2. Transcript levels in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ for 24 h. Fold changes in expression levels within a genotype are relative to the non-exposed plants (0 µM) (1.00 ± SE). Significant treatment effects within a genotype are highlighted: p<0.05 induction , repression ; p<0.01 induction , repression ; p<0.001 induction , repression . Genotype*treatment interaction effects are indicated in the last column of each treatment, and with bold font. The significant differences in fold changes between the two treatments within one genotype are indicated with: *p<0.05. Values are mean ± SE of four biological replicates.

LEAF	control		5 µM Cd			2 µM Cu			Differences fold changes Cd-Cu	
	wildtype	*vtc1-1*	wildtype	*vtc1-1*	Interaction	wildtype	*vtc1-1*	Interaction	wildtype	*vtc1-1*
$O_2^{\bullet-}$ scavenging										
MSD1	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.02	0.93 ± 0.04		1.02 ± 0.03	1.18 ± 0.07			
CSD1	1.00 ± 0.05	1.00 ± 0.03	**1.61 ± 0.17**	**0.69 ± 0.23**	**p<0.001**	0.83 ± 0.05	0.97 ± 0.08		*	
CSD2	1.00 ± 0.11	1.00 ± 0.04	0.38 ± 0.06	0.53 ± 0.12		0.36 ± 0.03	0.49 ± 0.08			
FSD1	1.00 ± 0.61	1.00 ± 0.37	0.47 ± 0.15	15.24 ± 6.10		1.01 ± 0.49	2.58 ± 1.14			
H_2O_2 scavenging										
CAT1	1.00 ± 0.08	1.00 ± 0.06	**2.13 ± 0.08**	**1.23 ± 0.21**	**p<0.05**	0.67 ± 0.06	0.79 ± 0.15		*	
CAT2	1.00 ± 0.01	1.00 ± 0.09	**0.25 ± 0.03**	**0.91 ± 0.11**	**p<0.001**	0.55 ± 0.04	0.84 ± 0.11		*	
CAT3	1.00 ± 0.07	1.00 ± 0.10	1.57 ± 0.19	1.46 ± 0.08		3.10 ± 0.17	2.58 ± 0.51		*	*
APX1	1.00 ± 0.05	1.00 ± 0.05	1.58 ± 0.26	1.30 ± 0.16		1.32 ± 0.13	1.10 ± 0.16			
APX2	1.00 ± 0.11	1.00 ± 0.07	**4.21 ± 0.60**	**0.87 ± 0.08**	**p<0.001**	**1.37 ± 0.09**	**0.65 ± 0.03**	**p<0.001**	*	
APX3	1.00 ± 0.01	1.00 ± 0.02	**0.60 ± 0.05**	**1.20 ± 0.08**	**p<0.001**	**0.69 ± 0.04**	**0.99 ± 0.06**	**p<0.01**		
APX4	1.00 ± 0.04	1.00 ± 0.04	**0.22 ± 0.03**	**0.70 ± 0.06**	**p<0.001**	**0.58 ± 0.08**	**0.87 ± 0.02**	**p<0.05**	*	
APX5	1.00 ± 0.10	1.00 ± 0.00	0.80 ± 0.08	0.85 ± 0.06		1.04 ± 0.06	1.10 ± 0.10			
APX6	1.00 ± 0.02	1.00 ± 0.06	**0.53 ± 0.05**	**0.98 ± 0.03**	**p<0.001**	**0.52 ± 0.05**	**0.74 ± 0.06**	**p<0.05**		
APX7	1.00 ± 0.05	1.00 ± 0.04	1.37 ± 0.19	1.51 ± 0.27		1.33 ± 0.07	1.11 ± 0.12			
sAPX	1.00 ± 0.04	1.00 ± 0.06	1.17 ± 0.20	1.02 ± 0.12		0.77 ± 0.07	0.81 ± 0.12			
tAPX	1.00 ± 0.02	1.00 ± 0.06	**0.42 ± 0.05**	**0.86 ± 0.04**	**p<0.001**	0.67 ± 0.08	0.81 ± 0.06		*	
MDHAR1	1.00 ± 0.03	1.00 ± 0.01	1.40 ± 0.10	1.20 ± 0.01		1.23 ± 0.04	1.28 ± 0.06			
MDHAR2	1.00 ± 0.08	1.00 ± 0.06	**13.16 ± 1.64**	**2.34 ± 0.39**	**p<0.001**	2.70 ± 0.17	1.71 ± 0.16		*	
MDHAR3	1.00 ± 0.58	1.00 ± 0.18	**76.13 ± 4.74**	**4.47 ± 0.23**	**p<0.001**	4.82 ± 0.55	1.93 ± 0.59		*	
MDHAR4	1.00 ± 0.06	1.00 ± 0.03	**0.60 ± 0.01**	**1.04 ± 0.05**	**p<0.001**	0.94 ± 0.05	0.99 ± 0.05		*	
MDHAR5	1.00 ± 0.01	1.00 ± 0.09	**0.43 ± 0.03**	**0.72 ± 0.03**	**p<0.001**	**0.40 ± 0.03**	**0.68 ± 0.02**	**p<0.001**		
DHAR1	1.00 ± 0.04	1.00 ± 0.01	**0.36 ± 0.02**	**0.77 ± 0.05**	**p<0.001**	0.67 ± 0.09	0.86 ± 0.09		*	
DHAR2	1.00 ± 0.04	1.00 ± 0.04	**2.69 ± 0.34**	**1.30 ± 0.10**	**p<0.001**	1.34 ± 0.09	1.23 ± 0.05		*	
DHAR3	1.00 ± 0.15	1.00 ± 0.03	1.44 ± 0.14	1.92 ± 0.16		3.92 ± 0.42	4.92 ± 0.37		*	*
GR1	1.00 ± 0.01	1.00 ± 0.06	**2.87 ± 0.12**	**1.37 ± 0.08**	**p<0.001**	1.08 ± 0.05	0.92 ± 0.09		*	*
GR2	1.00 ± 0.02	1.00 ± 0.06	**0.48 ± 0.03**	**1.07 ± 0.01**	**p<0.001**	0.88 ± 0.05	0.98 ± 0.11		*	
ROS production										
RBOHC	1.00 ± 0.17	1.00 ± 0.40	**313.99 ± 41.46**	**9.80 ± 3.06**	**p<0.001**	2.11 ± 0.30	1.87 ± 0.64		*	*
RBOHD	1.00 ± 0.10	1.00 ± 0.14	**3.07 ± 0.10**	**1.09 ± 0.14**	**p<0.001**	1.39 ± 0.13	0.99 ± 0.11		*	
LOX1	1.00 ± 0.06	1.00 ± 0.06	**2.43 ± 0.44**	**1.29 ± 0.05**	**p<0.01**	1.10 ± 0.01	1.43 ± 0.09		*	
LOX2	1.00 ± 0.04	1.00 ± 0.08	6.43 ± 0.56	4.73 ± 0.70		8.00 ± 0.17	5.07 ± 0.20			
Signaling										
OXI1	1.00 ± 0.13	1.00 ± 0.21	**112.18 ± 11.44**	**3.95 ± 1.62**	**p<0.001**	**3.07 ± 0.74**	**0.54 ± 0.15**	**p<0.01**	*	*
MPK3	1.00 ± 0.41	1.00 ± 0.19	3.86 ± 0.21	1.56 ± 0.32		3.11 ± 0.48	1.63 ± 0.26			
MPK6	1.00 ± 0.027	1.00 ± 0.03	**2.13 ± 0.08**	**1.55 ± 0.15**	**p<0.01**	0.98 ± 0.06	0.94 ± 0.02		*	*
WRKY25	1.00 ± 0.05	1.00 ± 0.20	**40.05 ± 2.31**	**2.91 ± 0.69**	**p<0.001**	**5.62 ± 0.72**	**1.33 ± 0.15**	**p<0.001**	*	
ZAT12	1.00 ± 0.42	1.00 ± 0.28	**19.39 ± 2.67**	**1.90 ± 0.57**	**p<0.01**	1.87 ± 0.49	0.79 ± 0.22		*	
HSF21	1.00 ± 0.34	1.00 ± 0.28	**17.19 ± 1.03**	**2.00 ± 0.45**	**p<0.001**	**5.35 ± 0.66**	**1.67 ± 0.30**	**p<0.05**	*	

6.3.5. Alterations in the expression of genes involved in oxidative signaling in A. thaliana wild-type seedlings versus vtc1-1 mutants after Cd or Cu exposure

In order to gather more information concerning the signaling in the different *A. thaliana* genotypes after Cd or Cu stress, the gene expression of H_2O_2-sensitive serine/threonine kinase OXI1 (oxidative signal-inducible 1), downstream MAPK cascade components and transcription factors was measured in both roots and leaves.

In roots, wildtypes and *vtc1-1* mutants showed an induced transcript level of *OXI1* in response to both metals (Table 6.1). In wildtypes, the expression of *MPK3* was increased after Cd stress and the expression of *MPK6* was decreased after Cu exposure, while no changes in *MPK* expression were observed in *vtc1-1*. Transcript levels of transcription factors *ZAT12* and *WRKY25* were upregulated in both genotypes in response to both metals, and *WRKY25* showed a significantly lower transcript level in *vtc1-1* compared to wildtypes after Cd and Cu exposure. Another H_2O_2-signaling gene is heat shock factor 21 (*HSF21*), the expression of which was induced in both genotypes after Cd exposure and only induced in wildtype when exposed to Cu.

In leaves, genotype differences were already observed under control conditions (Table 6.3), showing a significantly upregulated expression of *WRKY25* and *HSF21* in *vtc1-1* mutants. In Cd-exposed wildtypes (Table 6.2), all measured signaling genes, *i.e. OXI1*, *MPK3*, *MPK6*, *WRKY25*, *ZAT12* and *HSF21*, were upregulated, while this upregulation was absent or less pronounced in Cd-exposed *vtc1-1* plants. During Cu stress, increases were found in *MPK3*, *WRKY25* and *HSF21* in wildtypes, whereas no inductions were found in *vtc1-1* plants.

Table 6.3. Transcript levels in roots and leaves of 19-days-old *Arabidopsis thaliana* plants grown under control conditions in experiment 1 (A) and experiment 2 (B). Significantly different expression levels in mutants relative to the wild-type plants (1.00 ± SE) are indicated with colored font (induction **green** and repression **red**). Values are mean ± SE of four biological replicates.

A

EXPERIMENT 1		
ROOT	control	
	wildtype	*vtc1-1*
FSD1	1.00 ± 0.17	**10.15 ± 1.55**
DHAR3	1.00 ± 0.07	**0.76 ± 0.06**

LEAF	control	
	wildtype	*vtc1-1*
CAT2	1.00 ± 0.01	**0.68 ± 0.06**
MDHAR3	1.00 ± 0.58	**4.10 ± 0.73**
MDHAR5	1.00 ± 0.01	**0.56 ± 0.05**
WRKY25	1.00 ± 0.05	**5.50 ± 1.08**
HSF21	1.00 ± 0.34	**4.09 ± 1.14**

B

EXPERIMENT 2			
ROOT	control		
	wildtype	*vtc1-1*	*vtc2*
FSD1	1.00 ± 0.26	2.04 ± 0.38	0.82 ± 0.11
DHAR3	1.00 ± 0.13	**0.64 ± 0.02**	**0.56 ± 0.02**
ZAT7	1.00 ± 0.39	**3.54 ± 0.51**	1.67 ± 0.24

LEAF	control		
	wildtype	*vtc1-1*	*vtc2*
CAT2	1.00 ± 0.02	**0.78 ± 0.04**	0.93 ± 0.03
MDHAR3	1.00 ± 0.32	**4.97 ± 0.77**	3.15 ± 1.13
MDHAR5	1.00 ± 0.08	**0.70 ± 0.04**	**0.74 ± 0.02**
GSH2	1.00 ± 0.01	**0.76 ± 0.04**	**0.79 ± 0.02**
WRKY25	1.00 ± 0.12	**2.37 ± 0.29**	1.50 ± 0.26
HSF21	1.00 ± 0.09	**1.71 ± 0.25**	1.20 ± 0.15
ZAT7	1.00 ± 0.05	**7.76 ± 1.34**	**4.12 ± 1.14**
ACS2	1.00 ± 0.29	**5.62 ± 1.37**	**5.78 ± 1.59**

In general, the most pronounced difference between wildtype and the *vtc1-1* mutant was observed in leaves when plants were exposed to Cd. Moreover, the mutants showed a higher sensitivity to Cd as observed from the decreased fresh weights and increased TBArm levels found in Cd-exposed *vtc1-1* plants, while these changes were not observed in wildtypes. Furthermore, in *vtc1-1* plants, a diminished pro-oxidative NADPH-oxidase gene expression, a reduced expression of oxidative signaling genes, and a decreased response of antioxidative defense genes was measured. It is therefore interesting to further explore the role and the impact of AsA in the Cd sensitivity of plants, hence to find out whether AsA itself (*vtc2* mutants) or both the amount of AsA and the involvement in cell wall processes (*vtc1-1* mutants) are important during Cd exposure.

The response to Cd exposure investigated in *vtc1-1* and *vtc2*, two ASA deficient mutants with distinct properties regarding synthesis of GMP-derived GDP-sugar cell wall precursors

6.3.6. Plant growth in Cd exposed vtc1-1 versus vtc2 plants

When *A. thaliana* plants were exposed to Cd, a reduced root and leaf fresh weight was observed in *vtc1-1* mutants and not in wildtypes, which confirms the

observation in figure 6.4. However, no growth reduction was noticeable in *vtc2* plants (Figure 6.9A,B,C,D).

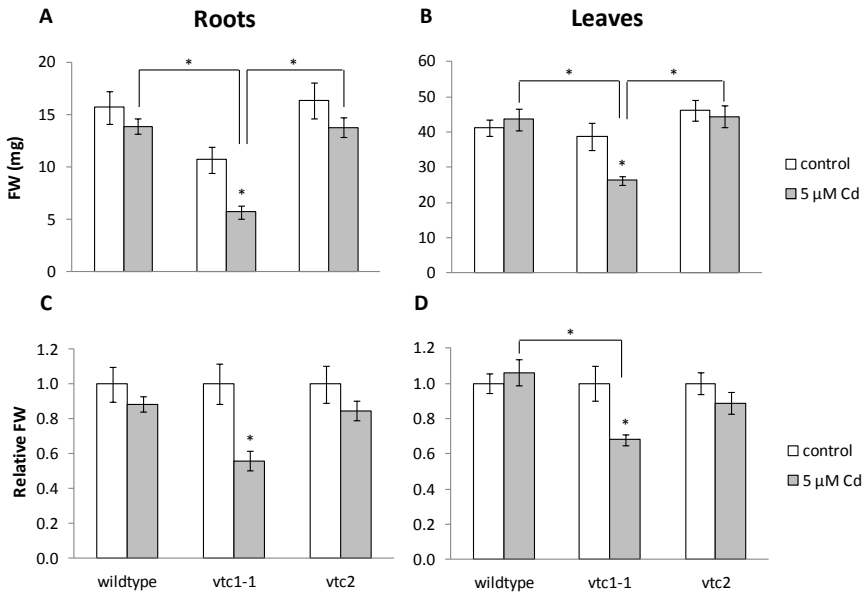

Figure 6.9. Absolute fresh weight (FW, mg) (A,B) and relative FW (C,D) of roots and leaves of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ for 24 h or grown under control conditions. Values are mean ± SE of at least 4 biological replicates. Significant differences relative to the unexposed genotype are shown: *$p<0.05$. The lines indicate a significant interaction effect.

6.3.7. The level of ROS production and membrane damage in the vtc mutants

Accumulation of H_2O_2 is a common response after exposure to Cd and was visualized by DAB staining in leaves of wildtypes and *vtc* mutants after 24 h exposure (Figure 6.10).

The H_2O_2 accumulation in the control group was negligible and no visible differences between genotypes were observed. An increased staining of the leaves in all genotypes after Cd exposure showed that H_2O_2 levels increased, but this increase was less pronounced in *vtc1-1* compared to wild-type and *vtc2* plants. These results indicated that wildtypes and *vtc2* are more exposed to Cd-induced H_2O_2 stress than *vtc1-1* plants.

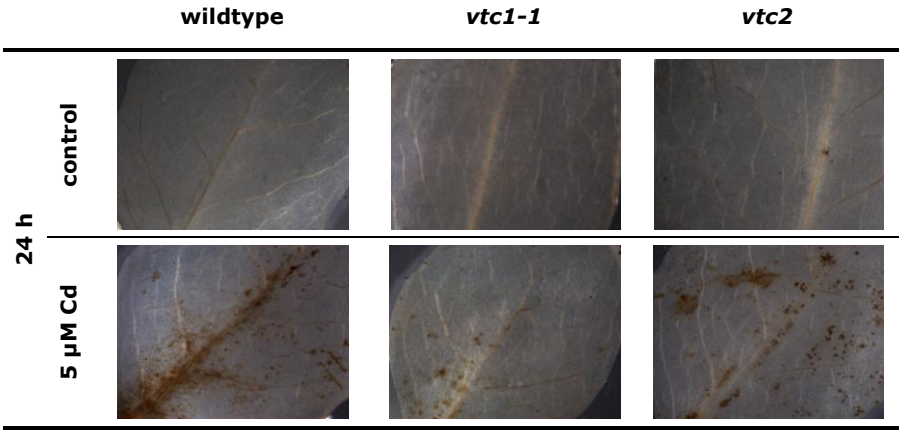

Figure 6.10. The leaves of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ for 24 h were stained with 3,3'-diaminobenzidine (DAB) to visualize the presence of H_2O_2. Brown spots were found in all three genotypes after exposure to Cd, but it was less pronounced in *vtc1-1* leaves.

To estimate the extent of membrane damage during Cd exposure, TBArm were determined in roots and leaves of the different genotypes. As in the previous experiment, a significant increase in lipid peroxidation was observed in the roots of the *vtc1-1* mutant and not in wild-type plants (Figure 6.11A,B). However, the *vtc2* mutant was similar to the wild-type and showed no increased lipid peroxidation in leaves or roots.

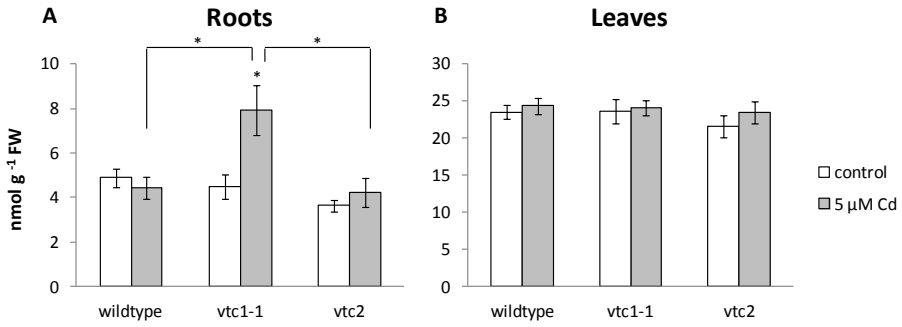

Figure 6.11. The concentrations of TBA reactive metabolites are expressed as nmol g^{-1} FW in roots (A) and leaves (B) of 19-days-old *Arabidopsis thaliana* genotypes exposed to 5 µM $CdSO_4$ for 24 h or grown under control conditions. Values are mean ± SE of six biological replicates. Significant differences relative to the unexposed genotype are shown: *p<0.05. Significant genotype differences are indicated with brackets. The lines indicate a significant interaction effect.

6.3.8. Alterations in the pro- and antioxidant gene network in vtc mutants

6.3.8.1. Pro-oxidative gene expression

In roots (Table 6.4), the expression of pro-oxidative genes *RBOHD* and *LOX1* was significantly increased in wildtypes after Cd exposure as shown before. Both *vtc* mutants failed to induce these genes to the same level as in wildtype. In wild-type leaves (Table 6.5), upregulations were found for *RBOHC*, *RBOHD* and *LOX1* after Cd exposure. Again, both *vtc* mutants did not show these inductions to the same level as wildtypes. Thus, the induction of pro-oxidative gene expression in roots and leaves in wildtypes after Cd exposure was absent or attenuated in both *vtc* mutants.

6.3.8.2. Antioxidative gene expression

In roots, under control conditions (Table 6.3), the expression of *DHAR3* was significantly lower in both *vtc* mutants compared to wildtypes and the expression of *FSD1* was two times higher in *vtc1-1* as compared to wildtypes (not significant). After Cd exposure (Table 6.4), the transcript levels of *CSD1* and *CSD2* were decreased in both *vtc* mutants but not in wildtypes. MicroRNA398 is known to regulate *CSD1* and *CSD2* transcript levels and the more pronounced decrease in *CSD1 and CSD2* expression in *vtc1-1* was concomitant with a strong increase in *miRNA398c* transcript level after Cd exposure that was not seen in wild-type and *vtc2* plants. Furthermore, all three *CAT* isoforms were expressed to a significantly lower level in *vtc1-1* compared to wildtypes after Cd exposure, which was also shown in the previous experiment (Table 6.1).

In leaves, under control conditions (Table 6.3), a higher expression of *MDHAR3* was observed, so although the gene was not (wildtype and *vtc1-1*) or not strongly (*vtc2*) upregulated under Cd stress, the resulting levels in the mutants are expected to be higher than in wildtypes. In contrast to the roots, the leaves showed more antioxidative gene expression differences between the two *vtc* mutants under Cd exposure (Table 6.5). In wildtypes exposed to Cd, a decrease was observed for the genes *CAT2*, *APX4*, *APX6*, *tAPX*, *DHAR1*, *MDHAR4*, *MDHAR5* and *GR2*. The same pattern was observed in *vtc2* mutants, but this decrease was stronger leading to a significantly lower expression compared to

Table 6.4. Transcript levels in roots of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 h. Fold changes in expression levels within a genotype are relative to the non-exposed plants (0 µM) (1.00 ± SE). Significant treatments effects within a genotype are highlighted: p<0.05 induction , repression ; p<0.01 induction , repression ; p<0.001 induction , repression .Genotype*treatment interaction effects are indicated with different letters (p<0.05). Values are mean ± SE of four biological replicates.

ROOT	wildtype		*vtc1-1*		*vtc2*	
	control	5 µM Cd	control	5 µM Cd	control	5 µM Cd
$O_2^{\cdot-}$ scavenging						
CSD1	1.00 ± 0.03	0.88 ± 0.06 **a**	1.00 ± 0.04	0.62 ± 0.03 **b**	1.00 ± 0.06	0.76 ± 0.05 **ab**
CSD2	1.00 ± 0.04	0.93 ± 0.06 **a**	1.00 ± 0.02	0.76 ± 0.03 **b**	1.00 ± 0.01	0.82 ± 0.04 **ab**
miRNA398b	1.00 ± 0.01	2.04 ± 0.12	1.00 ± 0.07	2.29 ± 0.28	1.00 ± 0.03	2.50 ± 0.17
miRNA398c	1.00 ± 0.41	2.70 ± 0.78 **a**	1.00 ± 0.24	215.59 ± 96.14 **b**	1.00 ± 0.42	0.99 ± 0.49 **a**
FSD1	1.00 ± 0.26	12.23 ± 2.23	1.00 ± 0.19	12.06 ± 1.62	1.00 ± 0.13	19.20 ± 4.58
H_2O_2 scavenging						
CAT1	1.00 ± 0.05	1.52 ± 0.04 **a**	1.00 ± 0.05	1.07 ± 0.04 **b**	1.00 ± 0.07	1.21 ± 0.09 **ab**
CAT2	1.00 ± 0.05	1.14 ± 0.06 **a**	1.00 ± 0.04	0.83 ± 0.06 **b**	1.00 ± 0.06	0.94 ± 0.06 **ab**
CAT3	1.00 ± 0.09	1.15 ± 0.06 **a**	1.00 ± 0.11	0.76 ± 0.06 **b**	1.00 ± 0.09	0.85 ± 0.09 **ab**
APX1	1.00 ± 0.18	0.83 ± 0.07	1.00 ± 0.08	0.67 ± 0.12	1.00 ± 0.12	0.75 ± 0.08
APX3	1.00 ± 0.07	1.08 ± 0.01	1.00 ± 0.03	0.91 ± 0.10	1.00 ± 0.06	1.11 ± 0.08
APX7	1.00 ± 0.11	0.72 ± 0.01	1.00 ± 0.04	0.87 ± 0.08	1.00 ± 0.03	0.80 ± 0.06
DHAR1	1.00 ± 0.07	1.16 ± 0.04 **a**	1.00 ± 0.01	0.76 ± 0.06 **b**	1.00 ± 0.09	0.96 ± 0.11 **ab**
DHAR2	1.00 ± 0.08	1.15 ± 0.05	1.00 ± 0.06	1.13 ± 0.03	1.00 ± 0.08	0.98 ± 0.04
DHAR3	1.00 ± 0.13	0.80 ± 0.02	1.00 ± 0.03	0.71 ± 0.13	1.00 ± 0.04	0.94 ± 0.16
MDHAR1	1.00 ± 0.08	1.15 ± 0.06 **a**	1.00 ± 0.17	0.61 ± 0.05 **b**	1.00 ± 0.19	0.62 ± 0.02 **b**
MDHAR2	1.00 ± 0.06	1.37 ± 0.05 **a**	1.00 ± 0.02	1.04 ± 0.05 **b**	1.00 ± 0.01	1.11 ± 0.03 **b**
MDHAR3	1.00 ± 0.05	1.07 ± 0.05	1.00 ± 0.04	1.26 ± 0.04	1.00 ± 0.05	1.01 ± 0.03
GR1	1.00 ± 0.03	1.20 ± 0.06	1.00 ± 0.02	1.03 ± 0.04	1.00 ± 0.07	1.06 ± 0.06
GR2	1.00 ± 0.02	1.06 ± 0.08	1.00 ± 0.00	1.00 ± 0.02	1.00 ± 0.04	0.88 ± 0.03
GSH1	1.00 ± 0.05	1.09 ± 0.05 **a**	1.00 ± 0.04	0.92 ± 0.03 **ab**	1.00 ± 0.07	0.81 ± 0.04 **b**
GSH2	1.00 ± 0.00	1.49 ± 0.05 **a**	1.00 ± 0.02	0.91 ± 0.05 **b**	1.00 ± 0.06	1.11 ± 0.08 **b**
ROS production						
RBOHC	1.00 ± 0.25	0.75 ± 0.07	1.00 ± 0.17	0.54 ± 0.16	1.00 ± 0.21	0.51 ± 0.08
RBOHD	1.00 ± 0.12	2.16 ± 0.16 **a**	1.00 ± 0.22	0.76 ± 0.07 **b**	1.00 ± 0.19	0.87 ± 0.12 **b**
LOX1	1.00 ± 0.10	4.07 ± 0.28 **a**	1.00 ± 0.07	1.22 ± 0.16 **b**	1.00 ± 0.08	1.86 ± 0.02 **b**
Signaling						
OXI1	1.00 ± 0.09	3.62 ± 0.98 **a**	1.00 ± 0.13	0.62 ± 0.14 **b**	1.00 ± 0.24	0.97 ± 0.19 **b**
MPK3	1.00 ± 0.08	2.29 ± 0.08 **a**	1.00 ± 0.05	1.10 ± 0.12 **b**	1.00 ± 0.13	1.48 ± 0.20 **b**
MPK4	1.00 ± 0.12	1.31 ± 0.10	1.00 ± 0.07	1.00 ± 0.10	1.00 ± 0.08	1.11 ± 0.05
MPK6	1.00 ± 0.10	1.08 ± 0.03	1.00 ± 0.06	0.86 ± 0.05	1.00 ± 0.09	0.99 ± 0.11
WRKY25	1.00 ± 0.13	2.07 ± 0.43	1.00 ± 0.12	1.07 ± 0.17	1.00 ± 0.15	1.62 ± 0.31
ZAT7	1.00 ± 0.39	13.21 ± 7.19 **a**	1.00 ± 0.15	2.52 ± 0.34 **b**	1.00 ± 0.14	4.42 ± 0.84 **b**
ZAT10	1.00 ± 0.09	4.46 ± 1.45 **a**	1.00 ± 0.08	1.71 ± 0.46 **b**	1.00 ± 0.13	2.09 ± 0.23 **ab**
ZAT12	1.00 ± 0.03	3.74 ± 1.25 **a**	1.00 ± 0.02	1.41 ± 0.26 **b**	1.00 ± 0.15	2.35 ± 0.36 **ab**
HSF21	1.00 ± 0.16	2.42 ± 0.47 **a**	1.00 ± 0.12	1.00 ± 0.22 **b**	1.00 ± 0.16	1.64 ± 0.36 **ab**
Genes involved in the ethylene biosynthesis						
ACS2	1.00 ± 0.12	2.48 ± 0.99	1.00 ± 0.06	1.70 ± 0.34	1.00 ± 0.01	2.12 ± 0.44
ACS6	1.00 ± 0.04	3.15 ± 0.60 **a**	1.00 ± 0.07	2.08 ± 0.17 **ab**	1.00 ± 0.03	1.89 ± 0.15 **b**

wildtypes. In contrast, these downregulations were not present in *vtc1-1* mutants. Furthermore, increased transcript levels of *CAT1*, *MDHAR2* and *MDHAR3* were observed in wildtypes exposed to Cd, also in *vtc2* plants but to a significantly lower level, while no such upregulations were detected in *vtc1-1* mutants.

In general, the most pronounced difference between the genotypes in antioxidative gene expression was observed in the leaves, with *vtc2* mutants showing stronger downregulations and less strong upregulations than in wildtypes, while *vtc1-1* mutants showed almost no alterations after Cd exposure.

6.3.9. Alterations in genes involved in oxidative signaling and ethylene biosynthesis in vtc mutants

In roots, a genotype difference was observed under control conditions for transcription factor *ZAT7*, of which the expression was significantly higher in *vtc1-1* as compared to wildtypes (Table 6.3). In Cd-exposed wild-type plants (Table 6.4), an increased expression was shown for most signaling genes, *i.e. OXI1*, *MPK3*, *WRKY25*, *ZAT*s, *HSF21* and for the genes involved in ethylene biosynthesis *ACS2* and *ACS6*. Of those genes, only *ZAT7* and *ZAT12* were upregulated in *vtc2* and *ACS6* in both *vtc1-1* and *vtc2*. As a result, in roots of *vtc1-1* plants no alterations were found in the expression levels of the measured signaling genes, while only *ZAT7* and *ZAT12* were responsive in *vtc2*.

In leaves (Table 6.5), all measured oxidative signaling genes, *i.e. OXI1*, *MPK*s, *WRKY25*, *ZAT*s, *HSF21*, and ethylene synthesis genes *ACS2* and *ACS6*, were upregulated in wildtypes exposed to Cd. The expression level of all these genes was in *vtc1-1* mutants either induced to a significantly lower level (*OXI1, WRKY25, ZAT10, ZAT12*, *HSF21*, *ACS2)* or no effect was observed (*MPK3, MPK4, MPK6, ZAT7*, *ACS6*). Also in *vtc2* leaves, some signaling genes and genes implicated in ethylene signaling were induced to a lower level as in wild-type plants, but the level of induction was mostly higher than in *vtc1-1* mutants. As a result, there was no significant difference in transcript levels of *MPK3*, *ZAT12* and *ACS6* between *vtc2* and wildtypes, but their levels were significantly lower in *vtc1-1*, while the transcripts of *ZAT7*, *ZAT10*, *HSF21* and *ACS2* in *vtc2* were expressed to the same level as *vtc1-1* plants. The level of induction of *OXI1, WRKY25* and *ACS2* in *vtc2* was between the level in wildtypes and *vtc1-1.*

Table 6.5. Transcript levels in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 h. Fold changes in expression levels within a genotype are relative to the non-exposed plants (0 µM) (1.00 ± SE). Significant treatments effects within a genotype are highlighted: p<0.05 induction, repression ; p<0.01 induction , repression; p<0.001 induction, repression. Genotype*treatment interaction effects are indicated with different letters (p<0.05). Values are mean ± SE of four biological replicates.

LEAF	wildtype		*vtc1-1*		*vtc2*	
	control	5 µM Cd	control	5 µM Cd	control	5 µM Cd
$O_2^{\bullet-}$ scavenging						
CSD1	1.00 ± 0.07	1.44 ± 0.08 **a**	1.00 ± 0.07	0.76 ± 0.08 **b**	1.00 ± 0.07	1.26 ± 0.22 **a**
CSD2	1.00 ± 0.06	0.87 ± 0.18	1.00 ± 0.10	0.64 ± 0.07	1.00 ± 0.09	0.51 ± 0.06
miRNA398a	1.00 ± 0.27	187.16 ± 32.67 **a**	1.00 ± 0.11	10.56 ± 4.05 **b**	1.00 ± 0.55	7.37 ± 2.43 **b**
miRNA398b	1.00 ± 0.25	3.52 ± 0.41 **a**	1.00 ± 0.25	3.90 ± 0.53 **a**	1.00 ± 0.21	0.92 ± 0.28 **b**
miRNA398c	1.00 ± 0.26	5.02 ± 0.65 **a**	1.00 ± 0.30	9.08 ± 0.69 **a**	1.00 ± 0.41	0.18 ± 0.12 **b**
FSD1	1.00 ± 0.31	1.19 ± 0.50	1.00 ± 0.35	2.79 ± 0.31	1.00 ± 0.33	6.44 ± 3.32
H_2O_2 scavenging						
CAT1	1.00 ± 0.12	1.80 ± 0.11 **a**	1.00 ± 0.09	1.29 ± 0.10 **b**	1.00 ± 0.06	1.68 ± 0.15 **ab**
CAT2	1.00 ± 0.02	0.38 ± 0.06 **a**	1.00 ± 0.05	0.85 ± 0.08 **b**	1.00 ± 0.03	0.39 ± 0.12 **a**
CAT3	1.00 ± 0.11	1.09 ± 0.11	1.00 ± 0.14	1.21 ± 0.12	1.00 ± 0.08	1.28 ± 0.16
APX1	1.00 ± 0.02	1.39 ± 0.14	1.00 ± 0.07	1.29 ± 0.07	1.00 ± 0.09	1.24 ± 0.06
APX2	1.00 ± 0.15	1.70 ± 0.10 **a**	1.00 ± 0.06	1.06 ± 0.08 **b**	1.00 ± 0.13	1.20 ± 0.22 **ab**
APX3	1.00 ± 0.04	0.75 ± 0.03 **a**	1.00 ± 0.06	1.03 ± 0.07 **b**	1.00 ± 0.01	0.52 ± 0.09 **c**
APX4	1.00 ± 0.09	0.36 ± 0.04 **a**	1.00 ± 0.06	0.74 ± 0.08 **b**	1.00 ± 0.05	0.08 ± 0.02 **c**
APX6	1.00 ± 0.06	0.75 ± 0.05 **a**	1.00 ± 0.02	0.92 ± 0.13 **a**	1.00 ± 0.04	0.27 ± 0.04 **b**
tAPX	1.00 ± 0.02	0.53 ± 0.04 **a**	1.00 ± 0.03	0.80 ± 0.14 **a**	1.00 ± 0.02	0.23 ± 0.06 **b**
DHAR1	1.00 ± 0.04	0.50 ± 0.02 **a**	1.00 ± 0.05	0.71 ± 0.11 **a**	1.00 ± 0.08	0.25 ± 0.03 **b**
DHAR2	1.00 ± 0.04	2.34 ± 0.04 **a**	1.00 ± 0.05	1.25 ± 0.12 **b**	1.00 ± 0.01	0.92 ± 0.08 **b**
DHAR3	1.00 ± 0.08	1.30 ± 0.22 **a**	1.00 ± 0.04	1.09 ± 0.04 **a**	1.00 ± 0.11	0.22 ± 0.02 **b**
MDHAR1	1.00 ± 0.02	1.05 ± 0.03 **a**	1.00 ± 0.06	1.10 ± 0.06 **a**	1.00 ± 0.03	0.41 ± 0.05 **b**
MDHAR2	1.00 ± 0.02	7.75 ± 1.09 **a**	1.00 ± 0.10	1.57 ± 0.14 **b**	1.00 ± 0.09	3.27 ± 0.61 **c**
MDHAR3	1.00 ± 0.32	64.21 ± 6.74 **a**	1.00 ± 0.16	4.24 ± 0.75 **b**	1.00 ± 0.36	13.92 ± 2.94 **b**
MDHAR4	1.00 ± 0.05	0.67 ± 0.04 **a**	1.00 ± 0.04	1.12 ± 0.04 **b**	1.00 ± 0.02	0.20 ± 0.03 **c**
MDHAR5	1.00 ± 0.08	0.59 ± 0.02 **a**	1.00 ± 0.06	0.95 ± 0.08 **b**	1.00 ± 0.03	0.17 ± 0.01 **c**
GR1	1.00 ± 0.02	2.22 ± 0.18 **a**	1.00 ± 0.06	1.07 ± 0.13 **b**	1.00 ± 0.03	1.24 ± 0.15 **b**
GR2	1.00 ± 0.02	0.69 ± 0.01 **a**	1.00 ± 0.05	0.91 ± 0.10 **b**	1.00 ± 0.02	0.49 ± 0.08 **a**
GSH1	1.00 ± 0.03	0.91 ± 0.08 **a**	1.00 ± 0.03	0.87 ± 0.06 **a**	1.00 ± 0.00	0.60 ± 0.03 **b**
GSH2	1.00 ± 0.01	1.93 ± 0.23 **a**	1.00 ± 0.05	0.77 ± 0.08 **b**	1.00 ± 0.03	0.68 ± 0.06 **b**
ROS production						
RBOHC	1.00 ± 0.31	61.63 ± 8.27 **a**	1.00 ± 0.09	1.32 ± 0.46 **b**	1.00 ± 0.64	1.56 ± 0.57 **b**
RBOHD	1.00 ± 0.05	3.96 ± 0.30 **a**	1.00 ± 0.06	1.41 ± 0.10 **b**	1.00 ± 0.03	1.50 ± 0.13 **b**
LOX1	1.00 ± 0.07	1.42 ± 0.12 **a**	1.00 ± 0.07	1.00 ± 0.09 **b**	1.00 ± 0.03	0.96 ± 0.04 **b**
Signaling						
OXI1	1.00 ± 0.07	76.04 ± 9.95 **a**	1.00 ± 0.08	3.97 ± 1.37 **b**	1.00 ± 0.12	20.14 ± 4.82 **c**
MPK3	1.00 ± 0.03	5.72 ± 0.33 **a**	1.00 ± 0.07	1.83 ± 0.34 **b**	1.00 ± 0.11	4.60 ± 0.60 **a**
MPK4	1.00 ± 0.05	2.54 ± 0.20 **a**	1.00 ± 0.03	1.39 ± 0.17 **b**	1.00 ± 0.06	1.12 ± 0.16 **b**
MPK6	1.00 ± 0.01	1.70 ± 0.10 **a**	1.00 ± 0.03	1.02 ± 0.11 **b**	1.00 ± 0.02	0.96 ± 0.10 **b**
WRKY25	1.00 ± 0.12	24.64 ± 2.83 **a**	1.00 ± 0.12	2.94 ± 0.64 **b**	1.00 ± 0.17	7.92 ± 1.35 **c**
ZAT7	1.00 ± 0.05	19.82 ± 2.78 **a**	1.00 ± 0.17	2.28 ± 0.39 **b**	1.00 ± 0.28	5.56 ± 1.55 **b**
ZAT10	1.00 ± 0.07	15.76 ± 2.36 **a**	1.00 ± 0.13	2.31 ± 0.07 **b**	1.00 ± 0.10	2.13 ± 0.56 **b**
ZAT12	1.00 ± 0.05	25.08 ± 2.73 **a**	1.00 ± 0.16	3.03 ± 0.41 **b**	1.00 ± 0.10	15.19 ± 3.54 **a**
HSF21	1.00 ± 0.09	18.31 ± 2.37 **a**	1.00 ± 0.15	2.36 ± 0.46 **b**	1.00 ± 0.12	4.57 ± 0.78 **b**
Genes involved in the ethylene biosynthesis						
ACS2	1.00 ± 0.29	568.12 ± 54.55 **a**	1.00 ± 0.24	2.74 ± 0.61 **b**	1.00 ± 0.28	25.14 ± 7.72 **c**
ACS6	1.00 ± 0.06	17.29 ± 2.30 **a**	1.00 ± 0.08	1.60 ± 0.28 **b**	1.00 ± 0.05	4.65 ± 1.13 **a**

6.3.10. The cellular redox state of AsA and GSH and enzyme activities compared between vtc1-1 mutants and wildtypes after Cd exposure

In general, when comparing the two *vtc* mutants after Cd exposure, the responses in the *vtc1-1* mutants were the most different from wild-type *A. thaliana* plants. A lower fresh weight, increased lipid peroxidation, and a lower H_2O_2 accumulation were observed in *vtc1-1* plants exposed to Cd, but not in the other two genotypes. Generally, the lack of induction of pro-oxidative genes, the lack of changes in antioxidative gene expression in the leaves, and a strongly attenuated induction of oxidative signaling genes was shown in both *vtc* mutants, but the effect was more pronounced in *vtc1-1* mutants. For this reason, it was interesting to examine whether a compensation mechanism for effects of low AsA levels existed in the form of (1) GSH as an antioxidant and (2) enzymes involved in the antioxidative defense system and lignification processes in *vtc1-1* mutants exposed to Cd.

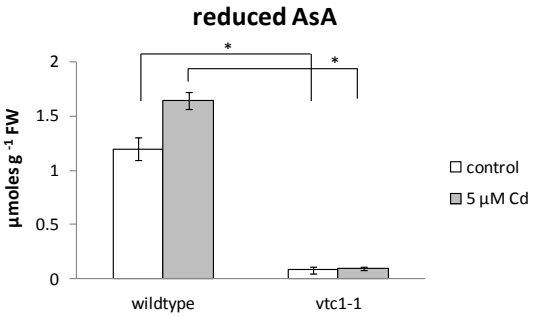

Figure 6.12. Reduced AsA (µmoles g^{-1} FW) in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 h or grown under control conditions. Significant genotype differences are indicated with brackets and *p<0.05.

The content of AsA and GSH, metabolites of the AsA-GSH cycle, was determined in leaves of *A. thaliana* wildtypes and *vtc1-1* mutants exposed to Cd for 24 h. As expected, reduced AsA levels were significantly lower in *vtc1-1* mutants compared to wildtypes (Figure 6.12). The amount of reduced GSH and total GSH were significantly higher in *vtc1-1* mutants under control conditions as compared to wildtypes (Figure 6.13A,B). After Cd exposure, the amount of total and reduced GSH was decreased in *vtc1-1* plants. The content of GSSG and the ratio GSSG/GSH were decreased after Cd exposure in both genotypes (Figure 6.13C,D). Moreover, the amount of GSSG was significantly lower in Cd-exposed *vtc1-1* compared to Cd-exposed wildtypes.

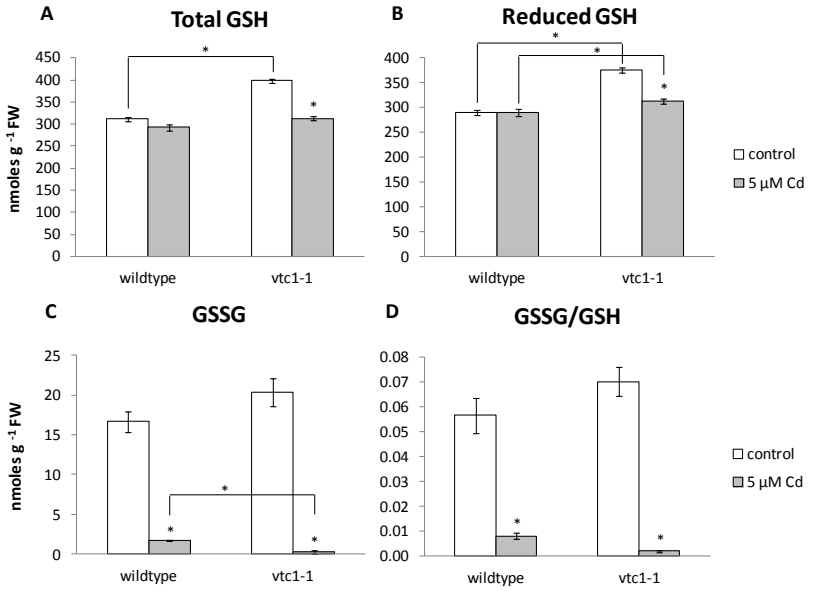

Figure 6.13. The contents of total GSH (A), reduced GSH (B), GSSG (C) and ratio GSSG/GSH (D) are expressed as nmoles g^{-1} FW in leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 h or grown under control conditions. Total GSH concentrations represent both reduced and oxidized forms; the redox state is the ratio between the oxidized and reduced form. Values are mean ± SE of at least six biological replicates. Significant differences relative to the unexposed genotype are shown: *p<0.05. The lines indicate a significant interaction effect.

The activities of antioxidative enzymes were measured in roots and leaves of 19-days-old *A. thaliana* plants exposed to Cd for 24 h (Table 6.6). Enzymes involved in the AsA-GSH cycle (APX and GR) were measured together with CAT, syringaldazine peroxidase (SPX), guajacol peroxidase (GPX) and SOD. Enzyme activities involved in NAD(P)H production were measured: malic enzyme (ME), isocitrate dehydrogenase (ICDH), glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In roots, genotype differences were found under control conditions. The activities of GPX, SPX and ME were significantly higher in *vtc1-1* mutants as compared to wildtypes. When plants were exposed to Cd, no significant changes were observed in wildtypes. However, in the *vtc1-1* mutant increases were shown in the activities of APX and CAT, and decreases were detected in SPX, ME and ICDH.

Also the leaves showed genotype differences under control conditions. A significantly higher activity was observed in the *vtc1-1* mutant for the enzymes APX, SOD, GR, ME and GAPDH as compared to wildtypes. In both genotypes exposed to Cd, increases were observed in GPX and CAT, the latter had a significantly higher activity in the *vtc1-1* mutant. Increases in the activities of SPX, GR and ME were shown in wild-type leaves exposed to Cd, while decreased activities of SOD and GAPDH were detected in Cd-exposed *vtc1-1* leaves.

Table 6.6. Activities of antioxidative enzymes in roots and leaves of 19-days-old *Arabidopsis thaliana* plants exposed to 5 µM $CdSO_4$ for 24 h or grown under control conditions. The enzyme activities are expressed as mU g^{-1} FW, except for APX and SOD that are expressed as U g^{-1} FW. Values are mean ± SE of at least 4 biological replicates. Significant differences in activity between unexposed genotypes relative to the wildtype are indicated with colored font (induction **green**). Significant differences for treatment effect (within one genotype) are highlighted: $p<0.05$ induction and repression, and significant interaction effects are shown with different letters ($p<0.05$).

ROOT	wildtype				*vtc1-1*			
	control		5 µM Cd		control		5 µM Cd	
APX	5.90 ± 0.32		6.03 ± 0.52	a	4.32 ± 0.25		10.18 ± 0.76	b
SOD	87.70 ± 19.58		86.78 ± 23.03		97.59 ± 3.85		123.99 ± 17.85	
GPX	157.71 ± 8.92		516.69 ± 165.58		**789.96 ± 122.88**		251.23 ± 32.17	
SPX	193.78 ± 70.14		450.84 ± 229.79		**1300.26 ± 243.15**		120.85 ± 38.54	
CAT	15.73 ± 1.52		15.23 ± 4.34		12.41 ± 2.81		28.03 ± 3.34	
GR	30.94 2.15		53.13 16.02		107.71 21.62		73.84 24.07	
ME	12.36 ± 1.67		30.00 ± 11.17		**76.93 ± 11.52**		20.02 ± 2.65	
ICDH	49.02 ± 30.46		17.46 ± 3.08		115.25 ± 40.40		10.92 ± 2.64	
G6PDH	7.03 ± 0.98		7.20 ± 3.39		12.08 ± 2.94		25.64 ± 8.88	
GAPDH	153.77 ± 16.20		185.87 ± 34.21		240.34 ± 45.36		269.74 ± 58.46	

LEAF	wildtype				*vtc1-1*			
	control		5 µM Cd		control		5 µM Cd	
APX	31.07 ± 3.54		32.92 ± 2.88		**60.50 ± 8.10**		62.95 ± 15.51	
SOD	326.03 ± 16.74		366.25 ± 7.22	a	**411.65 ± 13.28**		308.92 ± 9.16	b
GPX	41.27 ± 8.39		94.01 ± 8.64		44.44 ± 4.86		97.97 ± 16.75	
SPX	129.62 ± 26.33		364.05 ± 19.27	a	122.76 ± 9.38		142.92 ± 39.74	b
CAT	25.59 ± 4.82		47.56 ± 4.28	a	38.78 ± 4.42		81.63 ± 9.86	b
GR	947.02 26.09		1156.43 20.78		**1089.95 24.46**		1098.07 32.88	
ME	230.18 ± 7.22		597.42 ± 39.35	a	**426.63 ± 15.57**		479.70 ± 36.28	b
ICDH	488.73 ± 15.40		520.95 ± 21.13		555.53 ± 18.09		521.28 ± 33.56	
G6PDH	97.58 ± 7.39		130.48 ± 9.89		143.01 ± 5.51		156.24 ± 8.58	
GAPDH	584.96 ± 55.87		685.33 ± 77.41		**1011.53 ± 44.05**		554.32 ± 66.91	

6.4. Discussion

Ascorbate and its redox state can be involved in tuning cellular signaling pathways under (a)biotic stress conditions (Pignocchi and Foyer 2003b, Foyer and Noctor 2005b), but so far little has been revealed about its function when plants are exposed to Cd or Cu. Ascorbate deficient mutants *vtc1-1* and *vtc2* that are defect at different points in the AsA biosynthesis pathway (Figure 6.1) were available for this study. Under normal conditions, *vtc1-1* mutants had a shorter primary root when grown on vertical agar plates (Figure 6.6) and a smaller root fresh weight in hydroponics (Figure 6.4A), which was consistent with previous findings (Olmos *et al.* 2006, Kempinski *et al.* 2013). In addition, the leaves of *vtc1-1* showed a higher %DW (Figure 6.5) and an altered leaf morphology (Figure 6.3) compared to wildtypes under normal circumstances, which may point to AsA as an important component for normal growth and development. However, the AsA deficient *vtc2* mutant did not show a reduced root or leaf fresh weight under control conditions (Figure 6.9). This discrepancy may be explained by a second consequence of the *vtc1-1* mutation besides the decreased AsA level. In *vtc1-1* mutants, an inhibited activity of GMP (encoded on the VTC1 locus), results in a disturbed synthesis of GMP-derived GDP-sugar intermediates that are used in cell wall polysaccharides and glycoproteins necessary for normal growth (Smirnoff 2000b). Thus, the inhibited GMP activity and low AsA levels both prevented cell expansion early in development (Veljovic-Jovanovic *et al.* 2001, Olmos *et al.* 2006). Also, the activities of GPX and SPX were significantly increased in *vtc1-1* mutants as compared to wildtypes. Increased activities of cell wall peroxidases were also shown in the studies of Veljovic-Jovanovic *et al.* (2001) and Colville and Smirnoff (2008) studying *vtc1-1* mutants. Cell wall peroxidases have been implicated in determining cell wall structure and mechanical properties through peroxidative cross-linking of wall proteins and polysaccharides. In addition to the effect of increased peroxidase activity, a lower amount of AsA in the mutant, as mentioned before, could create an environment that markedly favors cross-linking (Veljovic-Jovanovic *et al.* 2001, Colville and Smirnoff 2008). Due to an increased cross-linking, reduced root growth and fresh weight were observed under normal circumstances in the roots of *vtc1-1* mutants and an altered leaf morphology can be explained.

Earlier research has established that an efficient use of the AsA-GSH cycle is necessary for normal plant physiology and it was suggested that the AsA pool exerts a profound influence on cellular redox signaling and plant growth responses (Pignocchi and Foyer 2003b, Foyer and Noctor 2005a, Foyer and Noctor 2005b, Pignocchi *et al.* 2006). Because the AsA pool has a role in cellular redox signaling, it might be involved in oxidative signaling required for the induction of defense responses against oxidative stress as a result of root metal uptake and/or translocation. To study whether AsA plays a regulatory role in the downstream responses of oxidative stress induced by metals, 19-days-old *vtc1-1 A. thaliana* mutant plants were exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$ and cellular responses were investigated after 24 h exposure. Because no genotype differences in Cd or Cu accumulation were detected (Figure 6.2), any differences in signaling and responses between the genotypes would be attributable to the low amount of AsA and/or an inhibited cell wall synthesis rather than a different stress level due to differential Cd or Cu contents. Once Cd or Cu is accumulated in plants, they can evoke various stress and defense responses (Yruela 2005, Gallego *et al.* 2012). In our study, the observed differences in response to metal exposure between wildtypes and *vtc1-1* mutants were more pronounced when plants were exposed to Cd as compared to Cu exposure. Whereas no clear differences in Cd uptake were observed, a higher level of stress was present in *vtc1-1* mutant plants as indicated by the reduction of leaf and root fresh weight and increased root lipid peroxidation, suggesting that this genotype is more susceptible to Cd exposure. The reduced root fresh weight in *vtc1-1* was concomitant with an increased %DW resulting from water loss due to membrane damage by lipid peroxidation. A similar explanation could be drawn for the roots of plants exposed to Cu, as Cu is well known to induce non-enzymatic lipid peroxidation due to the production of free radicals in Fenton and Haber-Weiss reactions. Another explanation for an increased %DW is the formation of a thicker cell wall by lignification (Gomes *et al.* 2011). An increase in lignin deposition after Cd exposure was observed before in different plant species and could be interpreted as a defense reaction that limits the entry of this metal or as a stress reaction that limits cell growth

Ascorbate and the activity of GMP interfere with Cd-induced oxidative signaling (Schützendübel *et al.* 2001, Ederli *et al.* 2004, Bhuiyan *et al.* 2007, Zagoskina *et al.* 2007).

As compared to wildtypes it was evident that *vtc1-1* mutants were more susceptible to Cd than when the plants were exposed to Cu and more differences in oxidative signaling were observed between the two genotypes after Cd stress. Therefore, the study further focussed on the investigation of the role and impact of AsA in the Cd sensitivity of plants. The *vtc1-1* mutant has a deficient activity of GMP involved in the biosynthetic pathway of AsA as well as in the synthesis of cell wall components (Wheeler *et al.* 1998, Smirnoff 2000b) (Figure 6.1). Because *vtc1-1* mutants were more sensitive to Cd exposure than wildtypes, and given the dual outcome of the mutation, a new question emerged: What is the contribution of AsA itself in the Cd sensitivity in plants? In an attempt to answer this question, the AsA deficient *vtc2* mutant was used. The *vtc2* mutant lacks an active GLGalPP in the biosynthesis of AsA, but this is in the pathway after the formation of GDP-mannose (Dowdle *et al.* 2007, Laing *et al.* 2007) (Figure 6.1). Cd-induced downstream responses at the cellular level were compared between *vtc1-1* and *vtc2* to investigate whether a low amount of AsA itself and/or the involvement in cell wall processes play an important role.
Exposure to Cd resulted also in this second experiment in a reduced root and leaf fresh weight and an increased lipid peroxidation (roots) in *vtc1-1* plants, in contrast to wild-type and *vtc2* plants showing no differences. This confirmed that *vtc1-1* mutants immediately suffered Cd stress not only due to a low AsA level but also to the decreased activity of GMP resulting in a decreased level of GDP-sugar intermediates, which are necessary for providing mechanical strength to cell walls and their expansion allowing the cell to grow and divide (Taiz and Zeiger 2010).

Previous studies demonstrated that H_2O_2 production in combination with the induction of NADPH oxisases (*RBOH*) and lipoxygenases (*LOX*) is clearly involved in Cd responses (Remans *et al.* 2010, Cuypers *et al.* 2011). In our study, in wild-type roots exposed to Cd, increased expression levels of pro-oxidative genes, *i.e. RBOHD* and *LOX1*, were observed while no differences or significantly lower expression levels were observed in both *vtc* mutants, especially in *vtc1-1*.

This indicates that in this mutant a lower H_2O_2 level might be present that can be further explained by increased APX and CAT activities under Cd stress. This in its turn led to a decreased oxidative signaling, which is represented in the expression levels of signaling genes, *i.e. OXI1*, *MPK3*, *WRKY25*, *ZAT*s, *HSF21* and *ACS2* and *ACS6*. In accordance with the studies of Opdenakker *et al.* (2012a) and Smeets *et al.* (2013) they were induced in wild-type plants but to a lesser extent in *vtc* mutants. Together with the decreased SPX activity, this indicates that both the concentration of AsA as well as cell wall processes in plants are likely to play a part in the intracellular coordination of the antioxidative system in response to Cd stress. Furthermore, in response to a reduced oxidative signaling, transcript levels of *CSD1* and *CSD2* were decreased in the roots of both *vtc* mutants, at least significantly for *vtc1-1*. This was associated with a great increase in the level of primary transcripts of the CSD1 and CSD2 regulating *miRNA398c* (Sunkar *et al.* 2006, 2007) in *vtc1-1* after Cd exposure, which was also seen in *vtc1-1 A. thaliana* plants during uranium stress (Saenen 2013). Furthermore, all members of the *CAT*-family showed a significantly lower expression level in *vtc1-1* roots as compared to wildtypes after Cd exposure. This reduced antioxidative defense expression could eventually contribute to the observed increased lipid peroxidation and decreased fresh weight in *vtc1-1* mutants.

In the leaves, exposure to Cd does not usually cause a significantly increased lipid peroxidation but it is known to cause an increase in H_2O_2 levels (Cuypers *et al.* 2011). Also in our experiments, no significant changes in lipid peroxidation were detected in the leaves of all genotypes, but increased stress levels were obvious from the increased H_2O_2 accumulation (Figure 6.10). However, *vtc1-1* mutants showed at this level a less pronounced H_2O_2 accumulation.
Some indications for the relatively lower level of H_2O_2 accumulation in the leaves of the *vtc1-1* genotype can be found in the gene expression data: first, this genotype generally has a decreased induction of pro-oxidative genes; second, they show a lack of upregulation of signaling components; third, the expression of anti-oxidative genes is responding differently to Cd stress, in particular the gene expression of catalase isoforms, producing bulk H_2O_2 scavenging capacity, is strongly inhibited in wild-type leaves but not in the *vtc1-1* mutant (Figure

6.8), leading to a compensatory increase in CAT activity in Cd-exposed *vtc1-1* mutants (Table 6.6).

This difference in H_2O_2 accumulation can at the same time be the cause of the observed changes in gene expression due to feedback and feedforward loops. Indeed, the production of ROS is not only involved in oxidative damage, it can also contribute to metal-induced signaling and acclimation responses (Quan *et al.* 2008). Both NADPH oxidases and lipoxygenases contribute to ROS production and are also involved in signaling responses of plant cells to (a)biotic stresses (Mithöfer *et al.* 2004, Bhattacharjee 2005). The activation of RBOHD is essential for signal propagation because accumulation of ROS is required for mediating cell-to-cell communication (Miller *et al.* 2009). Products of lipoxygenase activities can lead to the production of structurally different oxylipins (e.g. those of the jasmonate family) that are involved in inter- and intracellular signaling in multiple defense reactions also under Cd stress (Porta and Rocha-Sosa 2002, Kacperska 2004, Ali *et al*. 2005, Maksymiec and Krupa 2006b). The expression of *RBOHC*, *RBOHD* and *LOX1* was increased in wild-type leaves exposed to Cd (Remans *et al.* 2010, Cuypers *et al.* 2011), and indicated that pro-oxidative enzymes were induced as key components in Cd-dependent H_2O_2 production in wild-type leaves. Our data on the expression of pro-oxidative genes supports this notion that Cd can cause ROS production indirectly via pro-oxidative enzymes, which in turn results in activation of the OXI1-MAPK oxidative signaling cascades in roots and leaves (Table 6.1, 6.2, 6.4, 6.5), as was also observed by Opdenakker *et al.* (2012a). Both AsA deficient mutants had a diminished response at the expression level of pro-oxidative genes and oxidative signaling genes, with *vtc1-1* showing the strongest deviation from the wildtype.

Inhibition of antioxidative gene expression in the *vtc2* mutant leaves was generally stronger than in wildtypes, while these genes did generally not respond to Cd stress in the *vtc1-1* leaves (Table 6.5). Hence, despite the apparent lack of Cd-induced pro-oxidative gene expression, H_2O_2 accumulation in *vtc2* could be due to the strong inhibition of the antioxidative system, e.g. of genes involved in AsA-GSH cycle. In *vtc1-1* mutants, however, a significantly higher level of reduced and total GSH was found in the leaves of *vtc1-1* plants under control conditions, indicating that an increased GSH synthesis probably

counteracts the loss of AsA in this mutant (Veljovic-Jovanovic *et al*. 2001, Colville and Smirnoff 2008, Saenen 2013). This goes hand in hand with the more elevated activities of antioxidative enzymes such as APX, SOD, ME and GADPH (Table 6.6; Colville and Smirnoff 2008, Saenen 2013). Although *vtc1-1* mutants had a higher level of total and reduced GSH under control conditions, this level was negatively affected after Cd exposure, as well as the enzyme activities of SOD and GADPH.

In conclusion, the *vtc1-1* and *vtc2* mutants responded to Cd exposure differently from each other and from the wild-type plants. Cadmium stress resulted in reduced growth and membrane damage already after 24 h in *vtc1-1*, which was not noticed in the other genotypes, indicating that the involvement of GMP in cell wall processes was also important than AsA itself in provoking responses at this level (Figure 6.14). In *vtc1-1* mutants exposed to Cd, a reduced activation of pro-oxidative genes and a reduced accumulation of H_2O_2 in the leaves resulted in a strongly diminished oxidative signal transduction, a lack of response of antioxidative defense genes, and even decreases in enzyme activities as well as the GSH levels. The *vtc2* mutant leaves also lacked activation of pro-oxidative genes, but did show H_2O_2 accumulation and an oxidative signaling that was diminished but less strongly than in *vtc1-1.* Antioxidative defense genes were generally strongly inhibited specifically in the *vtc2* mutant, suggesting that a lack of AsA resulted in a signaling pathway that gave rise to decreased antioxidant responses after Cd exposure, leading to H_2O_2 accumulation.

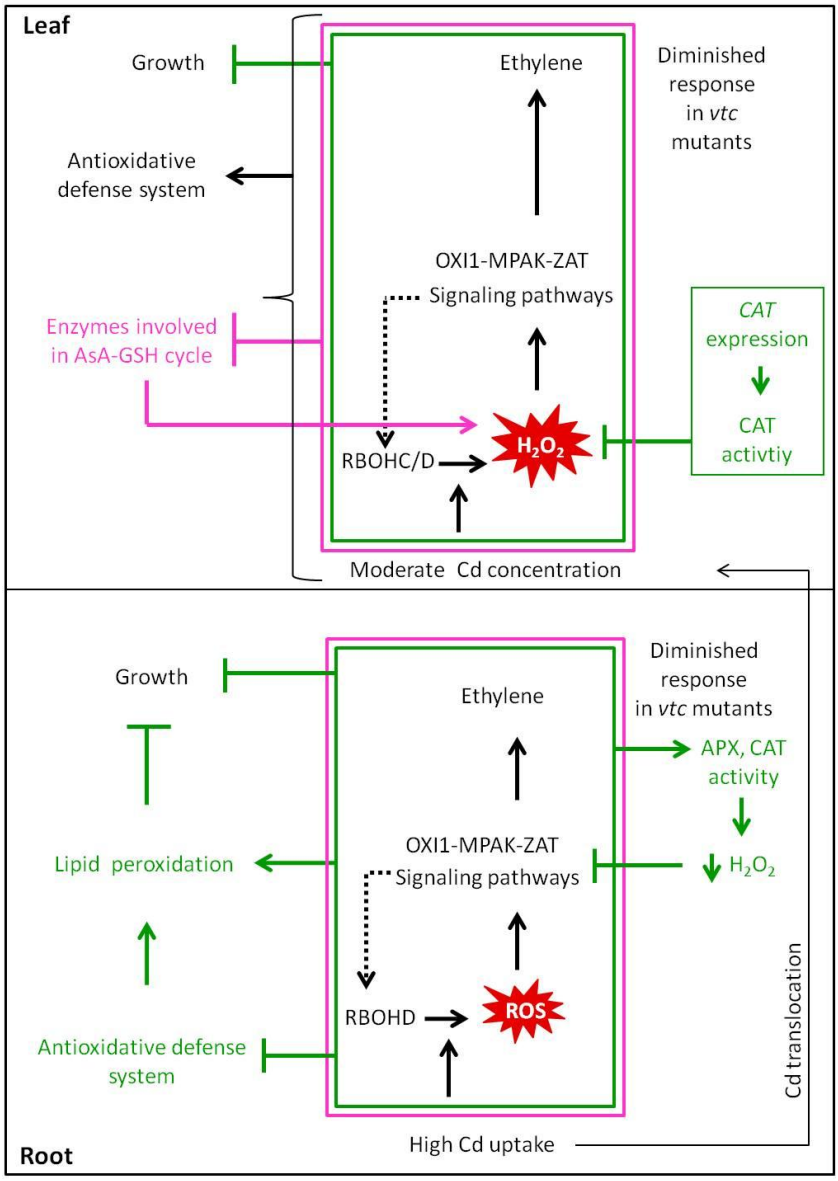

Figure 6.14. Overview of oxidative signaling pathways and defense responses in Cd-exposed *Arabidopsis thaliana* roots and leaves. Black lines represent responses in wildtypes, while different regulated responses in the mutants are represented by green lines (*vtc1-1*) and purple lines (*vtc2*). Dotted lines represent pathways found in literature.

CHAPTER 7
Long-term exposure of mutants to Cd or Cu

7.1. Introduction

Worldwide, toxic metals such as cadmium (Cd) and copper (Cu) have been dispersed in the environment due to industrial and agricultural activities. 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, Cu toxicity also remains a growing problem because of the high concentrations found locally in the environment (Sanità di Toppi and Gabbrielli 1999, Benavides 2005, Yruela 2005). Accumulation of these metals in plants disrupts physiological processes necessary for normal functioning and has negative consequences for the quality and safety of feed and food crops, thus causing serious concerns for human health (Islam *et al.* 2007, Bernard 2008, Cockell *et al.* 2008). At the cellular level, accumulation of toxic metals in plants induces a disturbance of the cellular redox state, which leads to an augmentation of reactive oxygen species (ROS). Even though the resulting increased ROS levels can cause cellular damage, controlled levels play an important role in modulating signaling networks that control physiological processes and stress responses (Mittler *et al.* 2004, Quan *et al.* 2008). Plants control ROS levels using their antioxidative defense system both under non-stress conditions, as well as under stress conditions such as exposure to excess metals. Ascorbate (AsA) is a well-known and important component of the plant's antioxidative system. As primary antioxidant, it can reduce ROS directly, or indirectly via ascorbate peroxidase (APX) in the ascorbate-glutathione (AsA-GSH) cycle (Noctor *et al.* 1998, Foyer and Noctor 2005a). In this cycle, enzymes and metabolites act together to detoxify H_2O_2 by the reducing power derived from NADPH (Foyer and Noctor 2005b, Halliwell 2006). The cooperation between AsA and APX is necessary to control H_2O_2 levels for signal transduction involved in many cellular processes. Levels and/or redox status of AsA, as well as the APX activity, may be influenced by metals, thereby disturbing oxidative signaling pathways that leads to altered cellular processes and defense responses in which these are

involved. Previous studies indeed showed altered activities and levels of APX and AsA after Cd or Cu exposure (Aravind and Prasad 2005, Lee *et al.* 2007a, Cuypers *et al.* 2011, Thounaojam *et al.* 2012). A role for APX and AsA in metal induced downstream responses was found in chapter 4, 5 and 6, and interesting differences in stress-related signaling and cellular responses were observed after short-term Cd and Cu exposure between the *Arabidopsis thaliana* wild-type, *ko-apx1* (*APX1* knockout), *ko-apx2* (*APX2* knockout) and *vtc1-1* (AsA-deficient) plants. The altered metal-induced short-term responses could eventually result in morphological and developmental changes, which may lead to an altered tolerance to metals. Our experimental aim was to identify and interpret in the context of plant survival the phenotypic effects in *A. thaliana* wild-type, *ko-apx1*, *ko-apx2* and *vtc1-1* plants that were from day 19 onwards continuously exposed to the non-lethal Cd or Cu concentrations used in the short-term exposure experiments. This could reveal a potential involvement of APX1, APX2 or AsA in plant development and metal tolerance under long-term Cd and Cu stress.

7.2. Experimental design and methodology

7.2.1. Plant material, treatment and phenotypic analysis

Seedlings were grown in diluted Hoagland hydroponic solution according to Smeets *et al.* (2008a), except that purified sand was used as a substrate instead of rock wool. The hydroponic culture was placed in a climate chamber with a twelve hour photoperiod at 65 % relative humidity and 22°C/18°C as day/night temperatures. Light was provided by a combination of blue, red and far-red LED modules (Philips Green-Power LED modules, The Netherlands) with a light intensity of 170 µmol m^{-2} s^{-1} at the rosette level. After 19 days, the hydroponic solution was supplied with 2 µM $CuSO_4$ or 5 µM $CdSO_4$, concentrations found in pore water of contaminated soils (Adriaensen *et al.* 2005, Krznaric *et al.* 2009). Throughout the entire time course of the experiment (day 19 to day 48), the plants were continuously exposed to Cd, Cu or grown under control conditions. New Hoagland solution with or without Cd or Cu was provided once every week to ensure and maintain nutrient availability (Semane *et al.* 2007, Dauthieu *et al.* 2009).

The measurements to assess the growth and development of unexposed and metal-exposed plants were started the day of exposing the plants to $CdSO_4$ or $CuSO_4$. Rosette growth was monitored every 3-4 days and as soon as inflorescence emerged, the height of the inflorescence per plant was also followed until a clear distinction between unexposed, Cd-exposed and Cu-exposed plants was observed (Boyes *et al.* 2001).

7.2.2. Statistical analysis

Statistics were performed on the endpoint measurements of number of leaves (day 36), leaf diameter (day 40) and inflorescence height (day 48). The datasets were analyzed using the two-way ANOVA in R (The R project for statistical computing) (R Development Core Team R 2008). Differences induced by treatment and between different genotypes were investigated using two-way ANOVA and Tukey post-hoc test. Normal distribution of the data and homoscedasticity were checked using respectively the Shapiro test and the Bartlett test. If necessary the data were transformed to achieve normality and homoscedasticity. When normality could not be reached, a non-parametric Kruskal-Wallis test was used followed by a Pairwise Wilcoxon rank sum test.

7.3. Results

7.3.1. Stress-induced vegetative changes in Cd- or Cu-exposed A. thaliana wildtypes versus mutants

Plants were grown for 19 days in control conditions and then exposed to 5 µM $CdSO_4$ or 2 µM $CuSO_4$. Signs of leaf chlorosis were visible in all genotypes after 4-5 days exposure to Cd (Figure 7.1) and worsened throughout the experiment. Interestingly, the *vtc1-1* mutant showed an earlier and more induced leaf senescence after Cd exposure as compared to the other genotypes (Figure 7.1). After 3 days exposure to Cu, leaves of all genotypes appeared purple (Figure 7.1), suggesting an accumulation of anthocyanins (Rauscher 2006, Misyura *et al.* 2012).

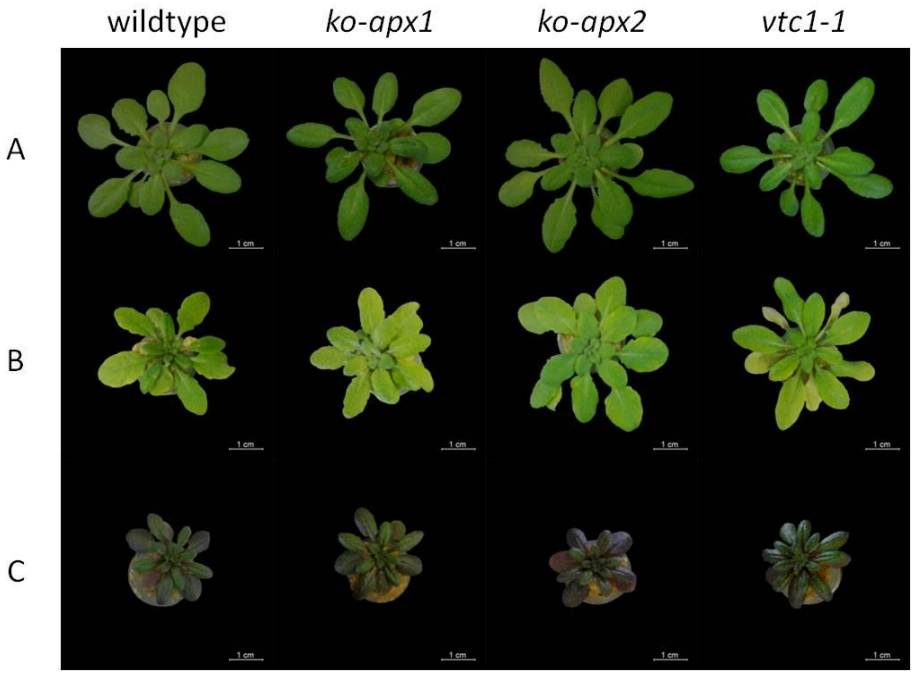

Figure 7.1. Representative pictures of the shoot appearance of *Arabidopsis thaliana* plants continuously exposed to 5 µM $CdSO_4$ (B), 2 µM $CuSO_4$ (C) or grown under control conditions (A). Pictures were taken at day 26, 5d after the start of exposure.

After Cd exposure, the number of leaves remained relative high, however a small significant decrease was noticed at day 36 for wild-type and *vtc1-1* plants exposed to Cd as compared to their control group, whereas all genotypes had a significantly smaller number of leaves after Cu exposure as compared to the control and Cd-exposed group (Table 7.1, Figure 7.2).

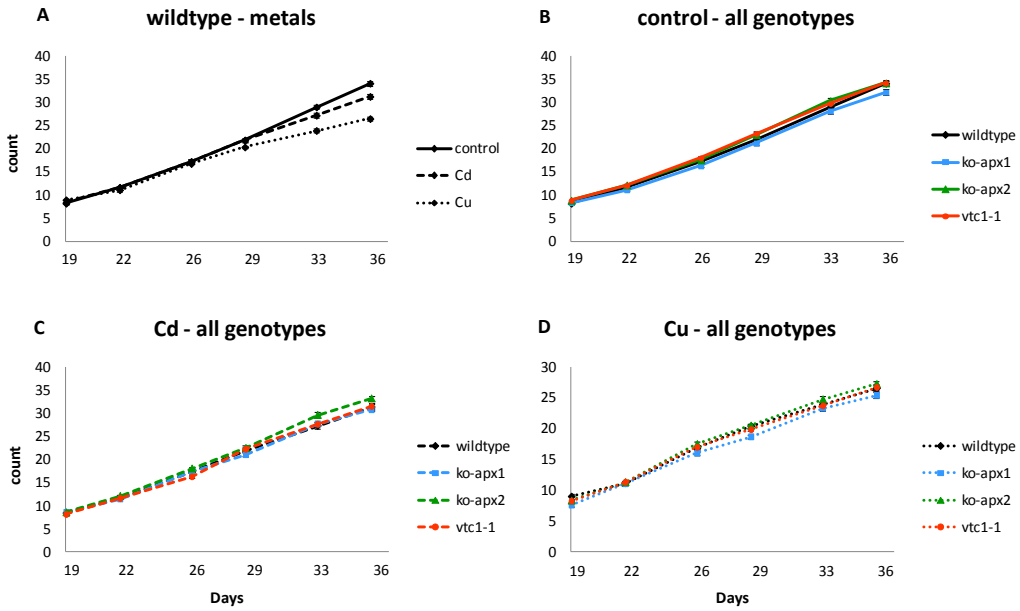

Figure 7.2. Number of leaves in *Arabidopsis thaliana* wild-type, *ko-apx1*, *ko-apx2* and *vtc1-1* plants continuously exposed to 5 µM $CdSO_4$, 2 µM $CuSO_4$ from day 19 onwards, or grown under control conditions. The average number of all leaves over time is shown; data are given as the average ± SE of 16 biological replicates per group. The number of leaves is represented in groups: (A) wild-type plants treated with all conditions (control, Cd and Cu), (B) all genotypes under control conditions, (C) all genotypes under Cd stress, (D) all genotypes under Cu stress.

Table 7.1. Endpoint data of number of leaves, rosette diameter (cm) and inflorescence height (cm) in *Arabidopsis thaliana* genotypes exposed from day 19 to day 48 to 5 µM $CdSO_4$, 2 µM $CuSO_4$ or grown under control conditions. Values are mean ± SE of 16 plants. Significant treatment effects within one genotype relative to the control group are highlighted ($p<0.05$; repression ■). Significant differences between the Cd-exposed and Cu-exposed group within one genotype are showed with *($p<0.01$) or **($p<0.001$). Genotype differences within one treatment (interaction effects) are represented by different lowercase letters.

	wildtype		*ko-apx1*		*ko-apx2*		*vtc1-1*	
Number of leaves (day 36)								
control	34.14 ± 0.56		32.21 ± 0.66		34.21 ± 0.57		34.21 ± 0.47	
5 µM Cd	31.29 ± 0.54	**ab** **	30.71 ± 0.34	**a** **	33.14 ± 0.55	**b** **	31.43 ± 0.64	**ab** **
2 µMCu	26.50 ± 0.42		25.36 ± 0.47		27.18 ± 0.44		26.64 ± 0.48	
Rosette diameter (cm) (day 40)								
control	9.96 ± 0.31	**a**	9.62 ± 0.22	**ab**	10.36 ± 0.26	**a**	9.05 ± 0.23	**b**
5 µM Cd	5.39 ± 0.17	**a** **	5.34 ± 0.16	**ac** **	6.08 ± 0.13	**b** **	4.91 ± 0.16	**c** **
2 µMCu	3.28 ± 0.06		3.45 ± 0.12		3.34 ± 0.10		3.16 ± 0.07	
Inflorescence height (cm) (day 48)								
control	18.31 ± 1.12	**ab**	15.62 ± 1.11	**a**	20.91 ± 0.99	**b**	20.21 ± 0.88	**b**
5 µM Cd	8.28 ± 0.98	**a**	11.00 ± 1.06	**a**	5.24 ± 0.59	**b** *	12.15 ± 1.04	**a**
2 µMCu	10.72 ± 0.70		8.38 ± 1.04		11.23 ± 0.91		9.14 ± 0.70	

The rosette diameter (Figure 7.3) was studied as morphological trait of vegetative growth and small differences were observed under control conditions. Endpoint measurements showed under control conditions a smaller rosette diameter for the *vtc1-1* mutant as compared to wild-type and *ko-apx2* plants, also *ko-apx1* rosettes were smaller as compared to *ko-apx2* plants (Table 7.1). Metal exposure clearly influenced the rosette diameter (Figure 7.1, 7.3 and Table 7.1). The average rosette diameter of all genotypes exposed to Cd decreased approximately with 50%, but this reduction was less pronounced in *ko-apx2* plants showing a higher rosette diameter throughout the entire experiment as compared to the other genotypes exposed to Cd. Figure 7.3 showed that the *vtc1-1* mutant initially had a larger diameter after Cd exposure as compared to wildtypes (at day 22 and 26; Figure 7.1) but they showed early a sign of growth inhibition so they were ultimately (at day 39) statistically smaller than wildtypes (Table 7.1). The difference in rosette diameter between unexposed and Cu-exposed plants was larger (approximately 70%), indicating that exposure to 2 µM Cu was more toxic than 5 µM Cd. Interestingly, *ko-apx1* and *ko-apx2* were initially less susceptible to Cu stress, but at day 26 they

exhibited a similar leaf growth inhibition as the other genotypes (Table 7.1, Figure 7.3).

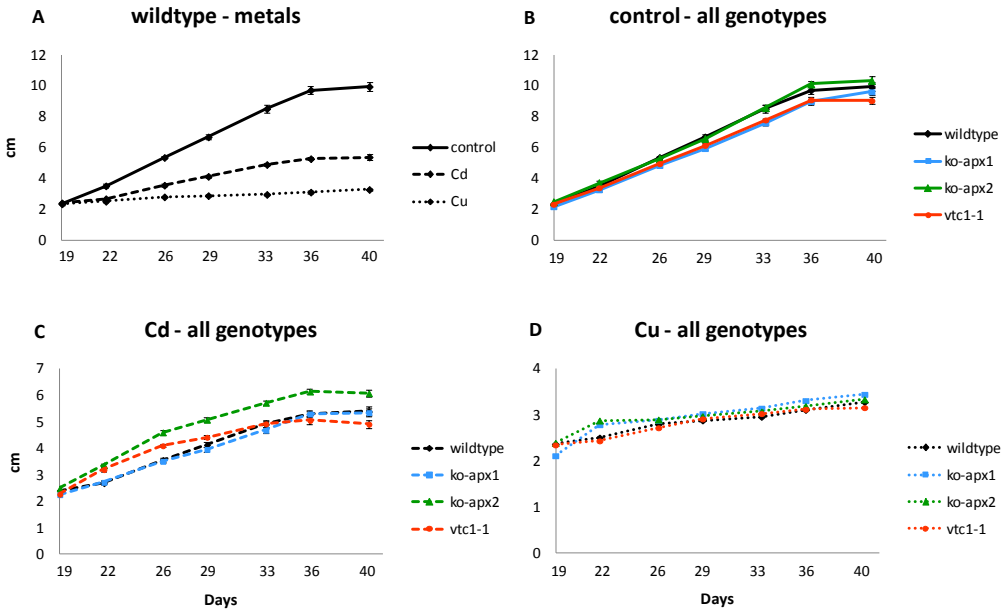

Figure 7.3. Rosette diameter of *Arabidopsis thaliana* wild-type, *ko-apx1*, *ko-apx2* and *vtc1-1* plants continuously exposed to 5 µM $CdSO_4$, 2 µM $CuSO_4$ from day 19 onwards, or grown under control conditions. The average rosette diameter (cm) over time is shown; data are given as the average ± SE of 16 biological replicates per group. The rosette diameter is represented in groups: (A) wild-type plants treated with all conditions (control, Cd and Cu), (B) all genotypes under control conditions, (C) all genotypes under Cd stress, (D) all genotypes under Cu stress.

7.3.2. Impact of metal exposure on inflorescence emergence in A. thaliana wildtypes versus mutants

The altered vegetative growth under metal stress conditions could have an impact on the time-point of emergence of the inflorescence meristem. Therefore the percentage of plants that started bolting was noted every day (Figure 7.4). Under control conditions, in *ko-apx1* mutants a small delay was observed in emergence of inflorescence as compared to wildtypes, while *ko-apx2* and *vtc1-1* plants showed a slightly faster occurrence (Figure 7.4B). When plants were

exposed to Cd, *vtc1-1* plants showed a later onset of bolting but the number of plants increased much faster as compared to the other genotypes (Figure 7.4C).

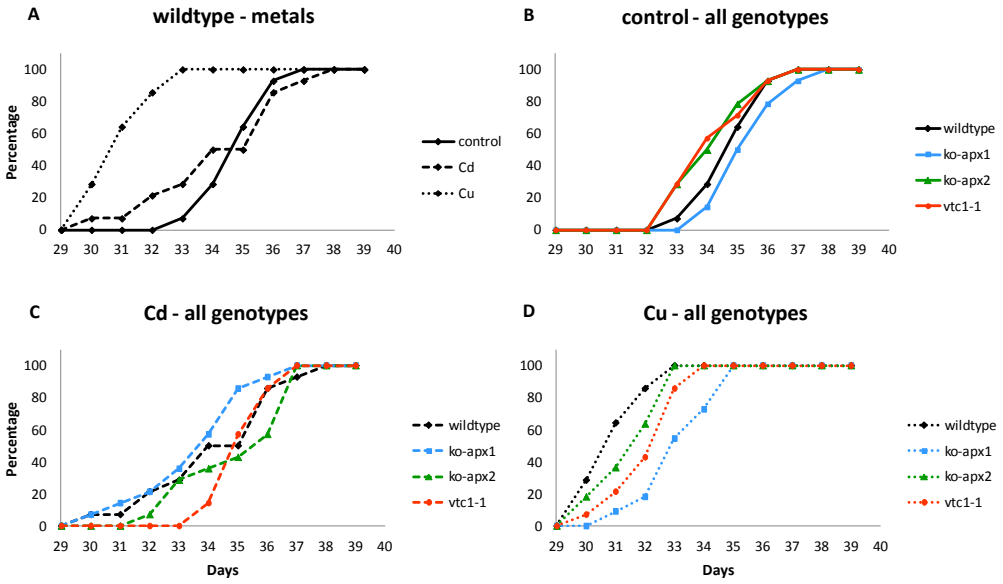

Figure 7.4. Inflorescence emergence in *Arabidopsis thaliana* wild-type, *ko-apx1*, *ko-apx2* and *vtc1-1* plants continuously exposed to 5 µM $CdSO_4$, 2 µM $CuSO_4$ from day 19 onwards, or grown under control conditions. The graphs represent the percentage of plants with inflorescence meristems over time and is represented in groups: (A) wild-type plants treated with all conditions (control, Cd and Cu), (B) all genotypes under control conditions, (C) all genotypes under Cd stress, (D) all genotypes under Cu stress.

Remarkably, inflorescences emerged earlier and faster in plants exposed to Cu as compared to the control and Cd-exposed group, which was definitely the case for wildtypes (Figure 7.4A). Although the mutants started to bolt around the same day as in wildtypes, reaching a 100% bolting stage took more time in *vtc1-1* and *ko-apx1* mutants.

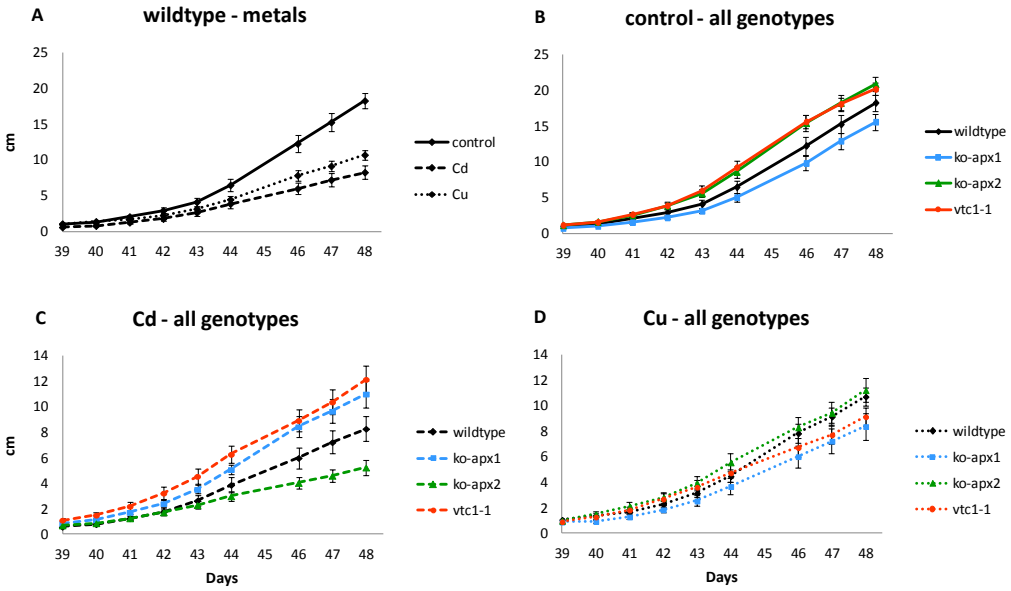

Figure 7.5. Inflorescence height of *Arabidopsis thaliana* wild-type, *ko-apx1*, *ko-apx2* and *vtc1-1* plants continuously exposed to 5 µM $CdSO_4$, 2 µM $CuSO_4$ from day 19 onwards, or grown under control conditions. The average inflorescence height (cm) over time is shown; data are given as the average ± SE of 16 biological replicates per group. The inflorescence height is represented in groups: (A) wild-type plants treated with all conditions (control, Cd and Cu), (B) all genotypes under control conditions, (C) all genotypes under Cd stress, (D) all genotypes under Cu stress.

Under normal circumstances, the inflorescences were significantly higher in *ko-apx2* and *vtc1-1* mutants as compared to *ko-apx1* plants. When plants were exposed to Cd and Cu, all genotypes showed significantly lower heights when compared to the control group (Table 7.1). Only significant differences between the Cd-exposed and Cu-exposed group were found in *ko-apx2* plants showing the highest inflorescence height after Cu exposure. Furthermore, a significantly higher length was observed for *ko-apx1* and *vtc1-1* as compared to *ko-apx2* mutants after Cd exposure (Table 7.1; Figure 7.5). The contrasting effect of Cd and Cu exposure on the mutants is remarkable: after Cd exposure *ko-apx1* and *vtc1-1* genotypes have longer inflorescences than wild-type while after Cu exposure these are shorter (Figure 7.5), for *ko-apx2* the opposite is observed.

7.4. Discussion

To incorporate cellular and molecular changes into a larger macroscopic context, we investigated the morphological effects of long-term exposure to environmentally realistic Cd and Cu concentrations using a phenotypic analysis. Two well-known effects frequently observed with prolonged metal exposure are chlorosis (Cd) and anthocyanin accumulation (Cu) (Van Belleghem *et al.* 2007, Collin *et al.* 2008, Keunen *et al.* 2011b), which are also found in our study. Moreover, leaf growth and leaf density were reduced by exposing *A. thaliana* to Cd or Cu confirming the studies of Keunen *et al.* (2011b) and MacFarlane and Burchett (2002). In our study it is clear that the applied concentration of Cu is more toxic than Cd, because of an observed growth inhibition (already at day 26), a well-defined decline in leaf density and a much earlier emergence of inflorescence meristems that is indicative for an accelerated senescence in the plants (Sandalio *et al.* 2001, El-Shora 2003). However, evidence is provided for the sublethal character of the applied Cd and Cu concentrations, which is revealed by the observation that all plants (in all conditions) were able to form inflorescence meristems, an indicative of the start of reproductive growth (Keunen *et al.* 2011b). Because the inflorescence height is associated with the number of siliques containing germinative seeds (Keunen *et al.* 2011b), plants exposed to Cd or Cu were considered to be less reproductive which was also found before (Keunen *et al.* 2011b, El-Shora 2003, MacFarlane and Burchett 2002). Continuously exposing *A. thaliana* to Cd or Cu is indeed reducing their reproductive capacity, but the plants are still able to grow, survive and produce siliques. However, further research is needed to elucidate whether there is a difference in seed production after exposure to Cd or Cu between the different genotypes.

What is the contribution of APX and AsA in the acclimation and survival of plants exposed to Cd and Cu? Concerning the vegetative growth, *ko-apx2* mutants were less sensitive to Cd, since the rosette diameter is large. However, the observed tolerance during vegetative growth was no longer noticed in the reproductive growth (inflorescence height), which was greatly reduced in Cd-exposed *ko-apx2* mutants. These results suggest that mechanisms and signals generated during Cd stress due to the absent of APX2 disturb

reproductive tissues indicating that APX2 is necessary for the protection of reproductive tissues when plants are exposed to Cd. However, during heat stress conditions an enhanced seed productivity in *ko-apx2* was observed due to an increased silique length (Suzuki *et al.* 2013). To find out the exact role of APX2 in reproductive tissues during metal stress further research is needed. Regarding the inflorescence height, there is a trend that *ko-apx1* and *vtc1-1* have more capacity for reproductive tissues, but further research is definitely essential.

Both *ko-apx* mutants immediately showed more tolerance to the applied concentration of Cu since a better vegetative growth was observed, suggesting that APX1 and APX2 are induced upon Cu-induced oxidative signaling that immediately resulted in growth inhibition. The observed higher tolerance in *ko-apx* plants was of short duration and ultimately growth inhibition was induced. Furthermore, a delayed emergence of inflorescence in all mutant plants exposed to Cu indicates a possible delayed stress signature in these mutants and an important role in the development of meristems especially for APX1 during Cu stress.

In general, continuously exposing *A. thaliana* wild-type, *ko-apx1*, *ko-apx2* and *vtc1-1* plants to Cd or Cu reduced their vegetative as well as reproductive growth, but they are all able to grow and survive. The *ko-apx2* mutant showed a stronger vegetative growth and a lower inflorescence height under Cd exposure, suggesting that the induction of this gene is necessary for increased reproductive capacity under Cd stress. Under Cu exposure on the other hand, the most delayed inflorescence emergence was observed in *ko-apx1* and *vtc1-1* plants, indicating these plants show a delayed stress signature as well as an involvement in maturation.

CHAPTER 8
General conclusion

8.1. Introduction

Worldwide, metal concentrations exceeding the natural emissions were found in the environment and cause significant losses in crop production. Besides growth retardation, plants exposed to metals show disruption of physiological processes, such as photosynthesis and respiration. Although the responses to toxic metal exposure in plants is quite similar on the macroscopic and physiological level, it is of great importance to expand our knowledge of the cellular and molecular processes influenced by metals. Oxidative stress is a common cellular consequence of toxic metal exposure. During oxidative stress a disturbance of the cellular redox balance, in favor of the pro-oxidants, leads to elevated concentrations of reactive oxygen species (ROS). Because the knowledge on underlying mechanisms and consequences of oxidative stress in plants exposed to metal toxicity is rather scarce, responses to copper (Cu) and cadmium (Cd) were investigated in this study. These two metals have different characteristics: Cu is an essential element necessary at micronutrient levels for normal plant development, while Cd is non-essential and toxic to plants even in low concentrations. In addition, Cu is redox-active and as such can induce ROS production directly, whereas the non-redoxactive Cd causes elevated ROS levels via indirect mechanisms. Increased ROS concentrations are harmful to the cell by damaging cellular components. However, tightly regulated levels of ROS are involved in signaling pathways controlling processes in normal metabolism and defense responses. A well-known example of ROS involved in the control of such processes and stress-related responses is hydrogen peroxide (H_2O_2). This molecule is immediately produced in plants exposed to Cd or Cu, thus H_2O_2 could be involved in metal-induced signal transduction events. Plant cells possess the ascorbate (AsA) - glutathione (GSH) cycle in which enzymes and metabolites cooperate to detoxify H_2O_2. The initial enzyme in this cycle is ascorbate peroxidase (APX) that reduces H_2O_2 using AsA as an electron donor. In the presence of AsA, APXs show a high affinity for H_2O_2, therefore APXs are

involved in protecting the plant cell against harmful amounts of H_2O_2 as well as controlling H_2O_2 levels for signaling involved in defense responses.

Since knowledge about stress signaling at the cellular level is necessary to understand plant responses to metals, the role of the AsA-related metabolism of the AsA-GSH cycle in oxidative signaling and its involvement in downstream Cu- and Cd-induced stress and defense responses was investigated in *Arabidopsis thaliana*. To this purpose, *ko-apx* (*APX* knockout) (Chapter 4 and 5) and vitamin C deficient (*vtc*) (Chapter 6) mutant *A. thaliana* plants were used and the observed responses were compared to those found in wild-type plants. Plants were grown and exposed to the metals in a hydroponic culture under controlled conditions, which has advantages as compared to soil systems, *i.e.* the environment (nutrients, temperature, light, humidity) was controlled to minimize other stress factors, and the concentrations of toxic metals could be easily applied to the roots in a homogenous way. The model organism *A. thaliana* has a relatively short life cycle and is easy to cultivate (Smeets *et al.* 2008a). Moreover, many scientists have been compiling phenotypical and molecular information about this plant into accessible databases (The Arabidopsis Information Resource), the complete genome sequence has been determined (The Arabidopsis Genome Initiative, 2000) and many mutants and transformants are available for functional genomics studies (European Arabidopsis Stock Centre). Therefore, this plant species has become an important research model in plant biology and genetics to study biochemical, cellular and molecular processes (Meinke and Cherry 1998).

8.2. The role of APX and AsA in Cd- and Cu-induced oxidative signaling and stress responses in roots of *A. thaliana*

After exposing *A. thaliana* wild-type roots to toxic concentrations of Cu or Cd, both metals were rapidly taken up by the roots. Because of the redox-active properties of Cu, it immediately induced oxidative stress as observed from induced gene expression levels of oxidative stress markers, an increased lipid peroxidation and an elevated percentage dry weight (%DW). A complete root growth inhibition was observed indicating that all plants clearly suffered Cu stress, however a root growth recovery was noticed in both *ko-apx* mutants,

indicating a role for APX1 and APX2 in Cu-induced growth retardation (Chapter 4). In contrast, prolonged (72 h) Cd exposure resulted in small reductions in root growth associated with small increases in %DW in wildtypes and both *ko-apx* mutants. However, *vtc1-1* mutants showed a higher sensitivity to Cd as indicated by the decreased root fresh weight and increased lipid peroxidation already after 24 h Cd exposure (Chapter 6). The differences in Cu- and Cd-induced morphological changes between the genotypes depend on alterations in oxidative signaling and hence different downstream responses.

Increased ROS production during Cu and Cd exposure was suspected because of enhanced transcript levels of NADPH oxidase (RBOHD) and due to the observed upregulations in oxidative signal-inducible kinase (OXI1) and redox-sensitive transcription factors, such as heat shock factor 21 (HSF21). Thus, an immediate activation of the OXI1-MAPK-ZAT cascade was observed at the transcript level, in response to the Cu- and Cd-induced oxidative challenge. This cascade is essential for the regulation and transcription of enzymes involved in pro- and antioxidative processes (Mittler *et al.* 2004) (Chapter 4, 5 and 6). During Cu stress the expression levels of the *RBOHD-OXI1* signaling pathway were still induced in wildtypes after prolonged (72 h) exposure, while no inductions were found in *ko-apx1* and *ko-apx2* mutants (Chapter 4). A similar expression pattern was found for the transcript levels of genes involved in the biosynthesis and signaling of ethylene (ACS2, ACS6, ERF1). Cu-exposed *ko-apx* mutants showed a suppressed signaling pathway and ethylene production, suggesting that the activity of APX1 and APX2 is needed to induce a proper signal transduction leading to the synthesis of ethylene. In turn, this may be responsible for the activation of a local auxin response leading to inhibition of cell elongation (Ruzicka *et al.* 2007), which may be linked to the fact that wildtypes suffered complete growth inhibition while in both mutants a small growth recovery was noticed (Figure 4.7; Chapter 4).

During Cd stress, both APX1 and GDP-D-mannose pyrophosphorylase (GMP/VTC1) activity in combination with AsA play a role in oxidative signaling. A diminished pro-oxidative NADPH-oxidase gene expression and a reduced expression of oxidative signaling genes were observed in Cd-exposed *ko-apx1* and *vtc1-1* mutants, probably due to decreased levels of H_2O_2 (Chapter 5 and 6). In *vtc1-1*, lower H_2O_2 levels may be explained by a compensatory

increase in CAT and APX activity. The suppressed signaling in turn gives rise to a decreased response of antioxidative defense genes and enzymes. As a consequence, APX1 is involved in the upregulation of APX and SOD activities since no elevations were observed in *ko-apx1* after Cd exposure (Chapter 5). A further role for AsA and GMP in the antioxidative defense responses after Cd exposure was evident from the *vtc1-1* plants showing a downregulation of *SOD*s, due to increased *miRNA398c* transcript levels, as well decreases in the activities of SPX, ME and ICDH. Thus, APX1, the level of AsA as well as cell wall processes in plants are likely to play a part in the intracellular coordination of the antioxidative system in response to Cd stress. On the other hand, APX2 was mainly involved in signaling and regulation pathways for the uptake and translocation of micronutrients after Cd exposure since more Zn content was observed in *ko-apx2* roots.

8.3. The role of APX and AsA in Cd- and Cu-induced oxidative signaling and stress responses in leaves of *A. thaliana*

Only a small part of the excess Cu-ions was transported to the leaves and an elevation of Cu content was seen after 24 h. At this time point, oxidative stress was evident from an increased expression of oxidative markers and an increased lipid peroxidation. After prolonged (72 h) Cu exposure, a growth reduction associated with an increased %DW was observed. Although Cu-exposed *ko-apx* mutants showed a similar level of lipid peroxidation as the wildtypes, both mutants seemed to acclimate and cope with Cu stress since higher fresh weights and unaltered %DW were found (72 h). This indicates that a lack of APX1 and APX2 resulted in altered signaling pathways leading to more adequate defense responses as compared to the wildtypes (Chapter 4). In contrast, Cd exposure neither caused growth reductions nor increased lipid peroxidation in both wild-type and *ko-apx* mutant plants, while *vtc1-1* plants were more susceptible to Cd stress as observed by lower fresh weight (already after 24 h). Although no oxidative damage was observed, Cd-exposed plants did show increased H_2O_2 levels. These levels were markedly lower in Cd-exposed *ko-apx1* and *vtc1-1* mutants (Chapter 5 and 6). As in roots, a compensation mechanism, *i.e.* increased CAT activity, could be the reason for lower H_2O_2 levels in *vtc1-1* leaves.

As in roots, the OXI1-MAPK-ZAT cascade is also activated in leaves after Cu and Cd exposure. Whereas there was no immediate accumulation of excess Cu in the leaves, genes involved oxidative signaling showed a fast (2 h) induction in wild-type and *ko-apx1* leaves, indicating the existence of root-to-shoot signaling (Chapter 4). The activity of APX2 is needed for an efficient root-to-shoot signaling after Cu exposure since a postponed signaling (RBOHD, OXI1-MAPK-ZAT) was found in *ko-apx2*, eventually leading to a delayed stress signature (ethylene and oxidative markers). The observed Cu-induced morphological differences between wildtypes and *ko-apx* mutants after 72 h may depend on changes in oxidative signaling and hence different downstream responses. *Ko-apx2* mutants were adapting to Cu stress via increased synthesis of antioxidative metabolites, primarily AsA. On the other hand, Cu-exposed *ko-apx1* leaves showed a constitutively higher expression of *ZAT7* during the entire exposure period, which could result in a repressed inhibition of defense responses causing the activation of plant defenses (other than we measured). These findings may be linked to the observed higher Cu tolerance in both *ko-apx* mutants (Chapter 4). However, on the long term, *ko-apx* mutants may not be more tolerant to Cu as they showed a similar inhibition of rosette growth and length of inflorescences as wild-type plants, even though a delayed onset of the inflorescence emergence was noticed (Chapter 7).

In Cd-exposed wild-type leaves, H_2O_2 levels increased and were sensed by *HSF21* - *ZAT12* - *APX1*, which in turn controls the transcript level of *RBOHD* essential for signal propagation. Also, the H_2O_2-sensitive OXI1-MAPK-ZAT cascade was activated leading to defense responses. As mentioned before, lower H_2O_2 levels were observed in *ko-apx1* and *vtc1-1* mutant plants. In *ko-apx1*, this could be due to alterations in root-shoot signaling as a result of suppressed root signal transduction affecting leaf responses in *ko-apx1*, such as altered ethylene biosynthesis, lower levels of GSH and lower antioxidant enzyme activities. This indicates that APX1 is involved and required for normal Cd-induced root-to-shoot signal transduction and leaf defense responses in *A. thaliana* (Chapter 5). In *vtc1-1* mutants, the lower H_2O_2 levels resulted in a strongly diminished induction of oxidative signaling genes leading to a lack of induction of pro-oxidative genes and a lack of changes in antioxidative gene expression. A low AsA level as well as a deficient GMP activity are thus both responsible for a

higher sensitivity of plants to Cd (decreased leaf fresh weight) and are both involved in Cd-induced signaling pathways (Chapter 6). Indeed after long-term Cd exposure, this resulted in a lower rosette diameter (Chapter 7). Although a decreased antioxidant defense response was noticed in *ko-apx1* and *vtc1-1*, both mutants showed similar inflorescences, even a small higher trend was observed, as compared to wildtypes after Cd exposure. It is tentatively suggested that *ko-apx1* and *vtc1-1* mutations triggered signaling pathways protecting reproductive tissues, but further research is essential.

On the other hand, APX2 influenced the nutrient profile as well as the metabolism of AsA (also seen during Cu stress), which are both strongly affected in *ko-apx2* after Cd exposure (Chapter 5). The upregulation of the AsA pool in *ko-apx2* mutants is needed to cope with Cd stress, and these plants showed more tolerance to Cd as indicated by a stronger vegetative growth. However, a lower inflorescence height was observed during Cd exposure, suggesting that APX2 is necessary for maintaining reproductive capacity (Chapter 7).

Finally, it is considered that the stimulation of Fe accumulation and SOD activity in both *ko-apx* mutants is needed for the protection of the chloroplasts to suppress light sensitivity in these mutants exposed to Cd (Chapter 5).

8.4. Conclusion

In general, exposing plants to 2 µM Cu seems to be more toxic than 5 µM Cd. In roots and leaves this was reflected by increased oxidative stress and membrane damage, and lower fresh weights after Cu exposure. However, similar inflorescence meristem lengths were observed after exposure to Cu and Cd, indicating they have a comparable reproductive capacity.

The aim of this study was to investigate the role of APX1, APX2 and AsA in oxidative signaling leading to stress responses after Cu or Cd exposure. During Cu stress, APX1 and APX2 are involved in signaling pathways leading to reduced growth in both roots and leaves, while a loss of function of APX1 or APX2 seems to result in more tolerance to Cu stress (72 h). The observed higher tolerance in both *ko-apx* plants was of short duration and ultimately growth inhibition was also induced.

During Cd stress, APX1 and AsA are important for the induction of signal transduction. A lower H_2O_2 accumulation was found in *ko-apx1* and *vtc1-1*

leading to decreased signaling and defense responses. Does this mean they show delayed stress signature or experience less stress? Regarding the vegetative growth, the *vtc1-1* mutant was more susceptible to Cd, while inflorescence heights in both mutants were similar, or a small higher trend was observed, as compared to wildtypes, but further research is necessary. Finally, a lack of APX2 results in a better vegetative growth, while APX2 is needed for the reproductive tissues upon Cd exposure.

8.5. Future perspectives

In plants, Cd and Cu induce an increase of the production of ROS and hence oxidative stress. Because ROS, more specifically H_2O_2, is known as a signaling molecule, it influences the expression of genes, which in turn has an influence at the metabolic level (Figure 8.1). The activation of enzymes and metabolites could result in plant acclimation to metal stress. During acclimation of the plant, APX and AsA play an important role. In the present study it was evident that APX and AsA in addition to their antioxidative capacities, both play a role in oxidative signaling during Cd and Cu exposure. To get a more profound insight in the Cd- and Cu-induced stress response mechanisms, it is attention-grabbing to study the underlying mechanisms of the gene expression (Figure 8.1). Firstly, it is interestingly to identify specific hormones and transcription factors that influence the regulation of the gene expression, which in turn could be affected by APX or AsA via signaling pathways. Secondly, gene expression is also regulated at transcriptional rate and/or posttranscriptional level, which is another research field that deserves more attention.

Another future perspective is to examine the tolerance of the mutants (*ko-apx1*, *ko-apx2* and *vtc1-1*) in more detail. Is there a difference in survival between leaves and roots? Is there a role for APX and AsA in the regulation of the reproductive tissues and/or in the quality and germination power of the seeds during metal stress?

Since polluted soils are most of the time contaminated with more than one metal, it is also interesting to include other metals, like zinc (Zn) for instance. Zinc is an essential non-redoxactive metal, that often occurs in Cd-contaminated areas. In this research it is interesting to investigate the role of multipollution in metal-induced oxidative signaling.

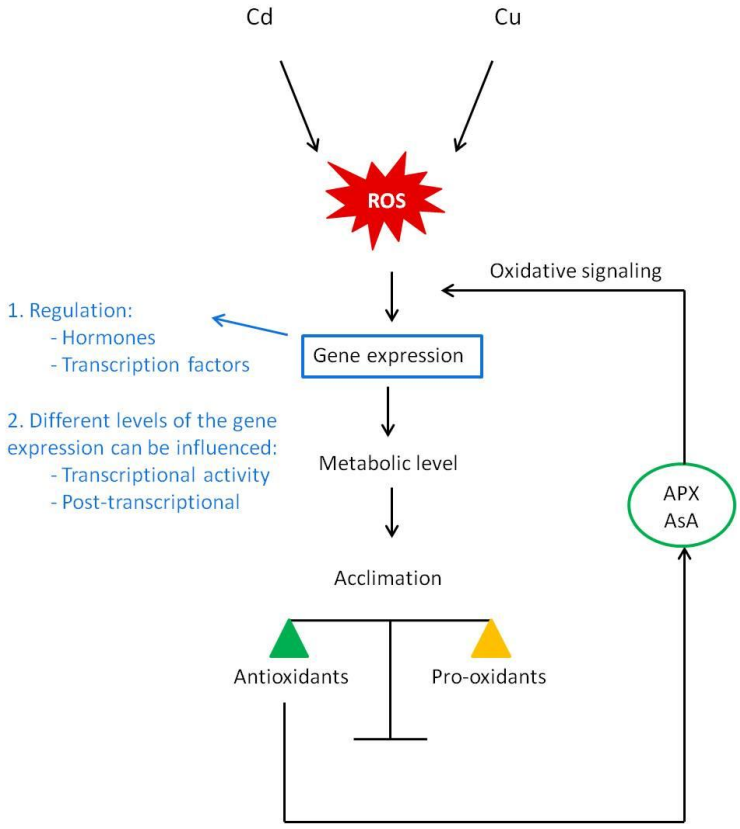

Figure 8.1. APX and AsA are involved in Cd- and Cu-induced oxidative signaling (black lines) which in turn could be involved in plant acclimation to metal stress. Further research is necessary (blue lines) to explore more details about the regulation of the gene expression.

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