2012 | Faculteit Wetenschappen

DOCTORAATSPROEFSCHRIFT

MAPKinases in cadmium or copper-mediated oxidative stress in *Arabidopsis thaliana*

Proefschrift voorgelegd tot het behalen van de graad van doctor in de wetenschappen, biologie te verdedigen door:

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D/2012/2451/38

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KNOWLEDGE IN ACTION

PhD thesis presented on the 2nd of October 2012 at Hasselt University

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Woord vooraf

Voila, hier staan we dan! Zes jaar geleden leek deze dag nog heel ver weg maar ik moet zeggen dat de tijd is omgevlogen en voordat je het goed en wel beseft, breekt die grote dag aan. Een doctoraat maak je echter niet alleen. Zonder de hulp van heel veel helpende handen, zou ik nooit zo ver geraakt zijn. Vandaar dat dit het uitgelezen moment is om iedereen, die er de afgelopen jaren voor mij was, te bedanken.

In de eerste plaats ben ik heel veel dank verschuldigd aan mijn promotoren: Ann en Jaco. Heel erg bedankt dat jullie in mij geloofden en mij deze kans hebben geboden. Het is door jullie dat mijn interesse voor de milieubiologie gewekt werd en ik de, in de ogen van mijn medestudenten, vreemde keuze maakte om mijn thesis binnen het CMK te maken. En dat beviel mij zo goed dat jullie niet meer van mij af konden geraken ☺.

Ann, op congres gaan met jou was een heel leerzame ervaring: van de Finse sauna over iets teveel cocktails drinken tot babbelen op de kamer tot een kot in de nacht ☺. Wat hebben we toch veel gelachen! Voor de mensen die nu denken dat een congres alleen maar vakantie is: er wordt ook gewerkt hoor! Ook bedankt om altijd met raad en daad klaar te staan voor mij, om mij te sturen, voor je enthousiasme als ik met nieuwe resultaten aan je deur stond, voor je geruststellende woorden (vooral in die laatste maanden) en je positieve commentaar op mijn papers … en waarschijnlijk vergeet ik nu nog veel dingen. En het MAPK-verhaal eindigt hier zeker en vast niet. Ik denk dat dit verhaal nog heel wat interessante denkpistes opent maar de opvolging is verzekerd (met Kerim).

Jaco, op congres gaan met jou was ook heel leuk: nog snel even langs het shoppingcentrum aan het einde van de congresdag of het altijd lekkere eten bij de Italiaan. Bedankt ook voor de altijd leuke commentaar op mijn resultaten (als ik deze doorstuurde of tijdens vergaderingen), voor het nalezen van mijn papers en de opbouwende commentaren, die het verhaal alleen maar beter maakten. Bedankt voor alles.

Een speciale dank je wel is er ook voor mijn collegaatjes van de oxidatieve stress groep, die altijd voor mij klaar stonden met een leuke babbel en zo veel meer. Toen ik hier begon als doctoraatsstudent, waren Karen S. en ik de enige doctoraatsstudenten in de groep. Ondertussen is de samenstelling van onze groep exponentieel gegroeid. In de eerste plaats is er Tony, onze postdoc. Tony, het was heel aangenaam om met jou samengewerkt te hebben. Door je grote kennis, die soms eindeloos lijkt, wist je altijd wel een manier om een probleem op te lossen en mij verder te helpen. Ook heel erg bedankt voor je opbouwende kritiek bij het nalezen van mijn papers / thesis. En natuurlijk zal ik je nooit vergeten als de sympathieke grapjas van het labo (soms weet ik toch echt niet of je nu meent wat je zegt of niet) en als de persoon die ons op zijn Australisch leerde feesten (wanneer is het volgende feestje?) ☺.

Dan Els, één van mijn eerste studentjes, die vond dat ik er best schattig uitzag op de foto op de UHasselt site en die toen dacht dat ik wel een leuke begeleider moest zijn ☺ (ik vraag mij af of ze dat nu nog steeds denkt). En Heidi, mijn buurmeisje op het werk, die zelfs zo ver gaat om speciaal voor mij gebakjes te gaan halen bij de bakker. Helaas kwam ze de dag erna met lege handen op het werk aan omdat haar schoonpapa ze had opgegeten. Toch bedankt hoor Heidi ☺. Meiden, allebei heel erg bedankt voor jullie steun, jullie advies, jullie vriendschap en zo veel meer. Ik ga jullie nooit vergeten en we houden wel contact via mail hé. Els, veel succes met jullie zoontje straks (en nee, ik ben niet verantwoordelijk voor die extra zwangerschapskilo's die erbij zijn gekomen door al die snoepjes te komen eten ☺) en natuurlijk ben ik één van de eersten om op bezoek te komen in het ziekenhuis. Heidi, hou mij op de hoogte van de broertjes of zusjes van Viktor die er zeker gaan komen en veel succes met de komende verbouwingen van jullie huis. Ik verwacht een uitnodiging voor de house warming ☺.

En dan is er ook nog mijn bureaugenootje Marijke. Bedankt voor de leuke tijd in de bureau, voor de leuke babbels en de afleiding, die vooral tijdens het schrijven van mijn doctoraat, met momenten zeer welkom was. Veel succes nog met jullie huisje en ik kom zeker eens kijken als het helemaal af is. Eline, jij ook bedankt voor de leuke momenten in de bureau tijdens je bezoekjes in de zomervakantie als het SCK gesloten was.

An, Kerim en Hanne, de laatste nieuwe aanwinsten van onze groep, het was super plezant om jullie erbij te hebben. Sacha, it was really nice to have you here. Good luck when you go back to Luxembourg. En aan al de rest van de groep, veel succes nog met jullie doctoraten. Aarzel niet om mij te contacteren als jullie nog met vragen zitten en hou mij op de hoogte van de afloop. Ik zal er zeker zijn op jullie verdedigingen!

Verder wil ik hier alle anderen, proffen, postdocs, doctoraatsstudenten, technisch personeel, kortom iedereen die deel uitmaakt of heeft uitgemaakt van de ondertussen serieus groot geworden biologiegroep, bedanken. Ieder van jullie heeft de afgelopen jaren wel ergens een rol van betekenis gespeeld in mijn doctoraatsleven, al was het maar om eens te informeren hoe het vlotte met mijn onderzoek. Iedereen persoonlijk bedanken zou mij veel te ver leiden en dan zou ik waarschijnlijk nog eens honderden pagina's aan dit boekje moeten toevoegen, daarom in het algemeen een welgemeend DANK JE WEL! Al wil ik een aantal mensen toch nog persoonlijk bedanken voor hun aandeel in mijn doctoraat.

Ann en Carine, zonder jullie zou het labo leeg zijn want jullie zorgen werkelijk voor alles wat wij maar nodig kunnen hebben. Jullie helpende handen kwamen altijd van pas en niets was jullie ooit teveel. Bedankt voor jullie geduldige en deskundige uitleg over honderd en één dingen en om mij op weg te helpen met de enzymmetingen en metaalbepalingen. Jullie waren ook steeds een grote hulp bij het zaaien en oogsten van mijn Arabidopsis plantjes en maakten er een echt feest van door de leuke babbels. Duizend maal bedankt!

Karen S., mijn ex-collegaatje. Jij introduceerde mij in de wereld van de real-time PCR en speelde een niet onbelangrijke rol in mijn beslissing om te gaan doctoreren. Sinds je verhuis naar boven, zien we elkaar spijtig genoeg niet veel meer. Bedankt dat ik altijd bij jou terecht kon voor een babbel of een portie goed raad. Joske, Michelle, Nele en Joke, jullie waren er ook van het begin bij. Bedankt voor jullie tijd, steun, goede raad en in het algemeen de leuke tijd die we hier samen hebben doorgebracht. Veel succes in jullie verdere carrière als prof/postdoc/of andere :-).

De ex-collegaatjes die het schip ondertussen al verlaten hebben. Karen V., zonder doctoraat was je waarschijnlijk nooit in mijn leven gekomen want wat heeft een mens nu te zoeken in het verre Zaventem (afgezien van het vliegtuig te nemen om op vakantie te gaan) ☺. Ik zal uw keuze om naar de UHasselt te komen nooit beklagen want het waren toch 4 leuke jaren samen. Lief en leed werden er gedeeld en onze tripjes naar Corfu en New York waren onvergetelijk, al was het maar ouw de kampeerfestijnen in uw kamer thuis aan de vooravond van ons vertrek. Sinds je het UHasselt schip hebt verlaten, mis ik onze roddeluurtjes en therapeutische sessies enorm. Gelukkig bestaat er nog zo iets als mail en is Harschot niet het einde van de wereld.
En dan zijn er ook nog altijd de dates van K3 met Anne, voor wie België niet groot genoeg was en die het geluk dan maar ging zoeken in Berlijn ☺. Ik zal nooit ons bezoek aan Berlijn vergeten. Het begon al met het buikgriepje van dat ene K3-lid, van wie ik de naam hier niet ga noemen ☺. Ik vraag mij eigenlijk nog steeds af of hij echt ziek was of gewoon schrik had om tussen drie vrouwen te moeten slapen ☺. En dan was er de verschrikkelijke koude en pakken sneeuw en ijs in Berlijn. Heel mooi allemaal maar ik zal de koude nooit meer vergeten. Een volgend bezoekje aan Berlijn zal zeker in de zomer vallen ☺. Nog veel succes daar in Berlijn, Anne, en hopelijk mogen we je in de toekomst terug in België verwelkomen.

Graag wil ik ook alle leden van de jury bedanken voor hun kritische evaluatie van dit proefschrift en hun opbouwende commentaar, die dit werk alleen maar beter heeft gemaakt. I would like to thank all members of the jury for their time and contribution to this PhD thesis: Prof. dr. Jan Colpaert, Prof. dr. Heribert Hirt, Prof. dr. Yves Guisez, Dr. Tony Remans and Dr. Henk Schat.

Het thuisfront, mama en papa, bedankt voor alle kansen die jullie mij gegeven hebben in het leven, voor het dak boven mijn hoofd en de hotelservice van de afgelopen jaren ☺. Binnenkort is het ermee gedaan. Wat gaan jullie dan doen met al die lege tijd? Stephanie en Glenn, jullie waren jaren lang goed voor mijn dagelijks soap met al jullie kot- en schoolperikelen (wie heeft er dan Thuis, Familie of Home and Away

nodig?) ☺. Maar toegegeven, zonder jullie zou het leven zo veel saaier zijn geweest. En iedereen komt uiteindelijk wel op zijn pootjes terecht. Stephanie, nog heel veel succes in je leven als dierenarts. En Glenn, misschien wordt je nog wel een echt goede boer ☺. Ook een grote dank je wel aan de rest van de familie, oma en opa, de tantes en nonkels, de nichtjes. Bedankt dat jullie er altijd voor mij waren en voor jullie interesse in de dingen die voor mij belangrijk zijn.

Mijn vriendenkliek (Evelien en Alex, Claudia en Kristof, Martijn en Liesbeth, Ilse en Reinhart), wat zou het leven saai zijn zonder jullie. Al zien we elkaar ondertussen wel een heel stuk minder dan vroeger toen we nog samen op de schoolbanken zaten. Verhuizen, nieuwe jobs, nieuwe interesses en kindjes, er blijft steeds minder tijd over om nog eens af te spreken ☺. Maar allemaal heel erg bedankt voor de leuke, fijne momenten (house warmings, vrijgezellen, trouwfeesten, de geboortes van de kindjes, …) die we de afgelopen jaren samen beleefd hebben. In het bijzonder Evelien en Claudia, die mij de laatste maanden van mijn werk weg sleurden voor onze ladies nights, dank je wel dames voor jullie steun en interesse. Het is supertof om gewoon eens vrijuit te kunnen zagen over onze mannen of het werk :-). Hopelijk is een nieuwe traditie geboren. En Martijn, heel veel succes met je doctoraatsverdediging binnen een paar weken en met jullie kleine spruit die onderweg is.

En als allerlaatste, de misschien wel belangrijkste persoon in mijn leven: Kristof, als ik niet was komen doctoreren, had ik je nooit leren kennen. Nog een pluspunt van mijn doctoraat ☺. Maar omdat je niet van melig gezever houdt, zal ik het hier kort houden ☺. Heel erg bedankt voor je steun (al was je misschien soms wel iets te veeleisend en hard voor mij) en geruststellende woorden (als ik het niet meer zag zitten) de afgelopen maanden. Ik zou niet weten wat ik zonder jou gedaan had. Dank je wel!

Kelly Opdenakker
Oktober 2012-09-05

Summary

Over the past decades, toxic concentrations of metals accumulated in soils by human activities, such as mining and industrial processes. In plants, metals interfere with essential physiological processes, like photosynthesis, transpiration and respiration, resulting in growth retardation and chlorosis. At the cellular level, metal exposure leads to an increased production of reactive oxygen species (ROS), thereby disturbing the cellular redox status and causing oxidative damage to cellular components. However, ROS can also act as signalling molecules in normal cell metabolism as well as in defence responses against biotic and abiotic stresses. In this way, ROS can interact with different signalling pathways in the cell, one of them being mitogen-activated protein kinase (MAPK) cascades. MAPK signalling pathways relay stress signals from the cell membrane to the nucleus by phosphorylation and activation of transcription factors, resulting in transcriptional control of stress-responsive genes. Although MAPK cascades are associated with copper (Cu) and cadmium (Cd) stress signalling, knowledge about their specific functioning in metal stress responses is rather scarce.

To understand plant responses to toxic concentrations of metals, it is necessary to gain insight in the specific stress signalling pathways operating at the molecular level thereby regulating cellular responses to these metals. Therefore, the main objective of this work was to unravel MAPK cascades controlling metal stress responses. For the metal treatment, two metals (Cu and Cd) with distinct characteristics (essential versus non-essential, redox-active versus non-redox-active) were used.

In the first part of this work (chapter 4), ROS production and the transcriptional induction of known MAPK components were investigated after metal exposure and compared between Cd- or Cu-exposed plants. In Cu-exposed roots, a fast induction of OXI1-MAPK cascades is observed, regulating transcription of pro- and antioxidative genes. The balance between these pro- and antioxidants regulates the ROS levels, which are increased upon Cu exposure. In addition, ROS produced after exposure to Cu induce lipid peroxidation leading to irreversible membrane damage, a condition that can be further enhanced by Cu-

induced lipoxygenase (LOX) activity. In contrast, Cd exposure of roots leads to a delayed induction of gene regulation via MAPK signalling characterized by no detectable H_2O_2 production nor lipid peroxidation. Since LOXs are induced by Cd exposure, their involvement in an altered oxylipin signalling is hypothesized. Leaves of Cu-exposed plants showed a fast as well as a delayed (biphasic) induction of the OXI1-MAPK signalling cascade, where the delayed induction may be the result of an altered oxylipin signalling. Previously, a biphasic antioxidant response to Cu was also described in leaves of Cu-exposed bean seedlings (Cuypers *et al.* 2000). Similar to the roots, Cd exposure of leaves resulted in a delayed induction of MAPK and oxylipin signalling.

In the following parts (chapter 5, 6 and 7), the function of the protein kinase OXI1 and the MAPKinase MPK6 in metal-induced oxidative stress was further investigated using *oxi1* and *mpk6* knockout *Arabidopsis* seedlings. In case of Cd exposure, no function for OXI1 and MPK6 in Cd uptake and translocation could be identified. On the other hand, OXI1 might be involved in Cu uptake and both OXI1 and MPK6 seem to be involved in Cu translocation from roots to shoots. Copper retention in the roots, which is normally seen in wild type plants after Cu exposure, was not observed in *oxi1* and *mpk6* mutant plants. After Cu exposure, these mutants accumulated higher Cu concentrations in their leaves in comparison to wild type plants, but no clear role could be attributed to phytochelatins (PCs) nor metallothioneins (MTs) in this process. Retention of Cu in the roots leads to oxidative stress in this organ while it protects the leaves from oxidative damage. Therefore, it was difficult to identify a role for OXI1 and MPK6 in controlling the cellular redox status after Cu exposure; changes in the redox balance are rather caused by the alterations in root and shoot Cu concentrations. However, metal-specific signalling cascades could be identified in Cu- or Cd-exposed roots and leaves.

In both Cu- and Cd-exposed roots and leaves, the OXI1-MPK6 signalling cascade was demonstrated to induce different signalling pathways by the activation of metal-specific transcription factors, resulting in different cellular stress responses. In addition, MPK6 was observed to play a role in the miRNA398-dependent regulation of Cu/Zn superoxide dismutase transcript levels after

metal exposure. In leaves, the OXI1-MPK6 pathway was also involved in LOX-induced oxylipin signalling. Both Cu and Cd exposure of *Arabidopsis* leaves resulted in an OXI1-MPK6 mediated stimulation of *LOX2*, possibly resulting in alterations at the level of oxylipin, more specifically jasmonic acid, production.

In conclusion, the results obtained in this work are indicative of an essential role for MAPK cascades in metal-induced oxidative stress responses. Depending on the chemical properties of the applied metal, the same MAPK cascade is able to regulate different metal-specific transcription factors resulting in metal-specific stress responses. Knowledge about the molecular mechanisms controlling metal stress responses is essential to understand how plants cope with elevated metal concentrations at the physiological level.

Samenvatting

De afgelopen decennia hebben er in de bodem toxische concentraties aan metalen geaccumuleerd als gevolg van humane activiteiten, zoals mijnbouw en industrie. In planten interfereren metalen met essentiële fysiologische processen, zoals fotosynthese, transpiratie en respiratie, met dwerggroei en chlorose tot gevolg en aldus een verminderde biomassaproductie. Op cellulair niveau induceert blootstelling aan metalen een verhoogde productie van reactieve zuurstofvormen (ROS), waardoor de cellulaire redox status verstoord wordt en oxidatieve schade aan cellulaire componenten kan ontstaan. Daarnaast hebben ROS ook een belangrijke functie als signaalmolecule zowel in het normale celmetabolisme alsook in verdedigingsresponsen tegen biotische en abiotische stressors. Op deze manier kunnen ROS interageren met verschillende signaaltransductie pathways in de cel, waaronder de 'mitogen-activated protein kinase' (MAPK) cascades. MAPK pathways geleiden stress signalen van het celmembraan naar de nucleus door fosforylatie en activatie van transcriptiefactoren, die de transcriptie van stressresponsieve genen controleren. Alhoewel MAPK-cascades worden geassocieerd met koper (Cu) en cadmium (Cd) geïnduceerde signaaltransductie, is er maar weinig geweten over hun specifieke functie in metaalgeïnduceerde responsen.
Om te kunnen begrijpen hoe planten reageren op toxische metaalconcentraties in hun omgeving, is het noodzakelijk om meer inzicht te krijgen in de specifieke stress signaaltransductie pathways op moleculair niveau die aan de basis liggen van de fysiologische responsen op deze metalen. Daarom was het belangrijkste doel van dit werk om de MAPK-cascades, die de metaalstress responsen controleren, te ontrafelen. Hiervoor werden twee metalen (Cu en Cd) met verschillende eigenschappen (essentieel versus niet-essentieel, redox-actief versus niet redox-actief) gebruikt.

In het eerste deel van dit werk (hoofdstuk 4) werden de ROS-productie en de transcriptionele inductie van gekende MAPK-componenten onderzocht en vergeleken tussen Cd- of Cu- blootgestelde planten. In Cu-blootgestelde wortels werd een snelle inductie van OXI1-MAPK cascades gezien, resulterend in de transcriptionele regulatie van pro- en anti-oxidatieve genen. De balans tussen

deze pro- en anti-oxidanten bepaalt het gehalte aan ROS, dat verhoogd is na blootstelling aan Cu. De Cu-geïnduceerde ROS-productie is verantwoordelijk voor een stijging in de lipidenperoxidatie, die nog verder verhoogd kan worden door de activiteit van lipoxygenasen (LOX). Als gevolg hiervan treedt er membraanschade op. Blootstelling van wortels aan Cd daarentegen leidt tot een vertraagde inductie van genregulatie via MAPK-signaling in de afwezigheid van waterstofperoxide (H_2O_2) productie en lipidenperoxidatie. LOXen worden ook geïnduceerd bij blootstelling aan Cd maar zouden hier eerder betrokken zijn in een veranderde oxylipinen signaling. Blaadjes van Cu-blootgestelde planten vertonen een snelle alsook een vertraagde (bifasische) inductie van de OXI1-MAPK signaalcascade, waarbij de vertraagde inductie waarschijnlijk het resultaat is van veranderingen in de oxylipinen signaaltransductie. Een bifasische respons in antioxidanten als reactie op blootstelling aan Cu werd reeds eerder beschreven in blaadjes van bonenplanten (Cuypers et al. 2000). Gelijkaardig aan de wortels, resulteerde Cd-blootstelling van de blaadjes in een vertraagde inductie van de MAPK en oxylipinen signaaltransductie.

In de volgende hoofdstukken (5, 6 en 7) werd de functie van het proteïne kinase OXI1 en het MAPKinase MPK6 in metaalgeïnduceerde oxidatieve stress verder onderzocht door gebruik te maken van *oxi1* en *mpk6* mutante *Arabidopsis* planten. Na Cd-blootstelling kon er geen rol voor OXI1 en MPK6 in de Cd-opname en translocatie aangetoond worden. OXI1 blijkt daarentegen wel betrokken te zijn in de opname van Cu en zowel OXI1 als MPK6 spelen een rol in de translocatie van Cu van de wortel naar het blad. Retentie van Cu in de wortels, zoals voorkomt in wildtype planten na Cu-blootstelling, werd niet opgemerkt in *oxi1* en *mpk6* mutante planten. Deze mutanten accumuleerden meer Cu in hun blaadjes na Cu-blootstelling in vergelijking tot wildtype planten. Toch kon er geen duidelijke rol worden toegeschreven aan fytochelatinen of metallothioneïnen in dit proces. Retentie van Cu in de wortels veroorzaakt oxidatieve stress in dit orgaan terwijl het de blaadjes beschermt tegen oxidatieve schade. Omdat veranderde Cu-concentraties in wortel en blad kunnen leiden tot veranderingen in de redox balans, was het moeilijk om een specifieke rol voor OXI1 en MPK6 in de controle van de cellulaire redox status na Cu-blootstelling te vinden. Metaalspecifieke signaaltransductie cascades daaren-

tegen konden wel geïdentificeerd worden in Cu- of Cd-blootgestelde wortels en blaadjes.

In wortels en blaadjes blootgesteld aan Cu of Cd, werd aangetoond dat de OXI1-MPK6-cascade verschillende signaaltransductie pathways kan induceren door metaalspecifieke transcriptiefactoren te activeren, resulterend in verschillende cellulaire stress responsen. Verder speelt MPK6 ook een rol in de miRNA398-afhankelijke transcriptie van Cu/Zn superoxidedismutase na blootstelling aan metalen. In blaadjes is de OXI1-MPK6-pathway ook betrokken in LOX-geïnduceerde oxylipinen signaling. Zowel Cu- als Cd-blootstelling van *Arabidopsis* blaadjes resulteerde in een OXI1-MPK6-gemedieerde stimulatie van *LOX2*, leidend tot veranderingen in de productie van oxylipinen, zoals jasmonaat.

Alles tesamen tonen de resultaten, bekomen in dit werk, aan dat MAPK-cascades een essentiële rol spelen in metaalgeïnduceerde oxidatieve stress responsen. Afhankelijk van de chemische eigenschappen van het toegediende metaal, is een bepaalde MAPK-cascade in staat verschillende metaalspecifieke transcriptiefactoren te reguleren, resulterend in metaalspecifieke stress responsen. Kennis over de moleculaire mechanismen, die metaalstress responsen controleren, is essentieel om te begrijpen hoe planten op fysiologisch niveau omgaan met verhoogde metaalconcentraties.

Table of contents

List of abbreviations

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
ANP1	*Arabidopsis* NPK1-like protein kinase 1
APX	ascorbate peroxidase
AsA	ascorbate
Ca	calcium
CAT	catalase
Cd	cadmium
CSD	Cu/Zn superoxide dismutase
CTR1	Constitutive Triple Response 1
Cu	copper
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DSP	dual-specificity phosphatase
EIN2	Ethylene Insensitive 2
EIN3	Ethylene Insensitive 3
ETR1	Ethylene Response 1
Fe	iron
flg22	bacterial flagellin peptide
FSD	Fe superoxide dismutase
GPOD	guaiacol peroxidase
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
H_2O_2	hydrogen peroxide
hn (CSD1/CSD2)	hetero-nuclear DNA (= primary transcript)
JA	jasmonic acid
LOX	lipoxygenase
MAPK/MPK	mitogen-activated protein kinase
MAPKK/MKK	MAPkinase kinase
MAPKKK	MAPkinase kinase kinase
MDA	monodehydroascorbate
MDAR	monodehydroascorbate reductase
MEKK1	MAPkinase kinase kinase
Mg	magnesium
miRNA398	microRNA398
MKP1	MAPK phosphatase 1
MKP2	MAPK phosphastase 2
Mn	manganese
MSD	Mn superoxide dismutase
MT	metallothionein
NDPK2	NDP kinase 2
$^1O_2$	singlet oxygen
$^3O_2$	molecular oxygen
$O_2^{\bullet-}$	superoxide radical
$^{\bullet}OH$	hydroxyl radical
OMTK1	Oxidative stress-activated MAP triple-kinase 1
OXI1	Oxidative-signal inducible 1
PA	phosphatidic acid
PC	phytochelatin
PCS	phytochelatin synthase
PDF1.2	plant defensin 1.2

PDK1	3-phosphinositide-dependent protein kinase 1
PLD	phospholipase D
pri-MIR398	primary transcript of MIR398
PrxR	peroxiredoxin
PSTP	protein serine-threonine phosphatase
PTI1	Pto-interacting 1, a protein kinase
PTP	protein tyrosine phosphatase
RBOH	respiratory burst oxidase homolog
ROS	reactive oxygen species
SA	salicylic acid
SOD	superoxide dismutase
SPOD	syringaldazine peroxidase
TBArm	thiobarbituric acid-reactive metabolites
WRKY22/25/29/33	WRKY transcription factor
ZAT12	zinc finger transcription factor
Zn	zinc

Chapter 1

Introduction

Kelly Opdenakker, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2012). MAPKinases in plant metal stress: regulation and responses in comparison to other biotic and abiotic stresses. International Journal of Molecular sciences, 13, 7828-7853.

1.1. Occurrence of cadmium and copper

Cadmium (Cd) and copper (Cu) are metallic elements, which have a relatively high density and are potentially toxic. Copper is an essential micronutrient required for normal plant growth and development (Marschner 1995). It can become toxic for organisms when its concentration exceeds the ones normally present in the environment. On the other hand, Cd is a nonessential element, which adversely affects biochemical reactions and physiological processes in plants (Cuypers *et al*. 2009). Cadmium and Cu are naturally occurring in the environment as a consequence of weathering of sedimentary rocks or are emitted into the atmosphere by eruptions of volcanoes or by forest fires. However, the major causes of the occurrence of toxic Cd and Cu concentrations in the environment are anthropogenic sources like mining and industrial processes. For example, Cu is emitted by the smelting of metals and Cd is produced as a by-product of zinc refining or coal mining. Cadmium and Cu are also released by energy-supplying power stations, such as coal burning, petroleum combustion and nuclear power stations, by combustion of diesel engine and lubricants during transportation of vehicles, and by incineration of municipal wastes. In addition, Cd and Cu are also used in the processing of plastics, textiles, electronic devices, Ni-Cd batteries, wood preservation and paper processing. The metal content of soils can also be increased due to the agricultural use of phosphate fertilizers, metal-containing pesticides, fungicides and sewage sludge, or irrigation with wastewater (Nriagu and Pacyna 1988, Kirkham 2006, Nagajyoti *et al*. 2010).

Uptake and accumulation of these metals by food and feed crops brings these potentially toxic metals into the food chain for consumption by animals (cattle) and humans (Chary *et al*. 2008).

1.2. Effects of cadmium and copper in humans

Generally, Cd concentrations in ambient air are low, contributing only to a few percent of the total absorbed dose of Cd in the body. However, tobacco leaves accumulate significant amounts of Cd making tobacco smoking an important source of Cd exposure (Järup and Akesson 2009). In the non-smoking population, dietary intake of Cd is the main source of environmental Cd exposure. However, Hogervorst *et al*. (2007) reported that house dust is a potentially important route of Cd exposure in areas with contaminated soils. Health effects of Cd were already reported in the 19th century. Persons using Cd-containing polishing agent displayed acute gastrointestinal symptoms as well as delayed respiratory symptoms (Nordberg 2009). In humans, Cd has a biological half-time of 10-30 years and is efficiently retained in the kidney. As a consequence, Cd is nephrotoxic and can initially cause kidney tubular damage, eventually leading to renal failure after prolonged and/or high exposure (Järup and Akesson 2009). Cadmium can also cause bone damage, which was identified for the first time in Japan shortly after World War II. People eating Cd-contaminated rice, suffered from the so-called Itai-Itai disease, characterized by osteomalacia, osteoporosis and kidney damage (Nordberg 2009). Recently, Nawrot *et al*. (2010) demonstrated that healthy men, which are occupationally exposed to Cd via the air, showed a lower bone mineral density and a higher urinary calcium excretion leading to a higher risk of developing osteoporosis. In 1993, Cd was classified as a human carcinogen (group I) by the International Agency for Research on Cancer (IARC) (Järup and Akesson 2009). A significant association between exposure to Cd and the risk of developing lung cancer was reported for a population environmentally exposed to Cd released by non-ferrous smelters in the past (Nawrot *et al*. 2006). In addition, Cd is also associated with prostate, renal, endometrial and breast cancer (Järup and Akesson 2009).

In the population, Cu is mostly taken up via the consumption of food and drinking water. Especially drinking water can be a major source of Cu for humans because of the use of Cu plumbing systems. Cu uptake via inhalation or dermal routes is rather negligible. Acute toxicity can result from the ingestion of excess Cu and is characterized by stomach distress causing nausea and vomiting. The effects of chronic Cu toxicity are best studied in Wilson disease,

an autosomic recessive disorder, leading to Cu accumulation in the liver, brain and cornea. Therefore Wilson patients suffer from chronic liver disease accompanied by neurological or psychiatric impairment, kidney malfunction, or ophthalmologic, haematological and skeletal manifestations. Other examples of chronic Cu toxicity are Indian Childhood Cirrhosis and Idiopathic Chronic Toxicosis. These diseases were related to the consumption of food stored or prepared in Cu-containing utensils and caused severe liver damage leading to death. Since the use of Cu-containing utensils was abandoned in 1974, no further cases were observed (de Romaña *et al*. 2011). Copper is also an essential metal in cell division processes in normal tissues and consequently increased serum Cu levels seem to be associated with prostate, breast, colon, lung and brain cancer. In addition, Cu seems to be involved in neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis, Parkinson disease and Alzheimer disease (Matés *et al*. 2010).

1.3. Effects of cadmium and copper in plants

1.3.1. Metal uptake and transport

Plants can take up Cd and Cu from the soil or water through their roots. Uptake directly from the atmosphere via the leaves is rather scarce (Dalcorso *et al*. 2008). The degree to which plants are able to take up metals depends on their concentration in the soil and their bioavailability. The latter is modulated by several soil characteristics, like the presence of organic matter, pH, redox potential, temperature, concentrations of other elements, the occurrence of plant-associated microorganisms, and the plant species. Apart from the bioavailable fraction of metals in the soil solution, uptake activity and translocation efficiency also determine the plant's metal uptake. The cell wall contains binding places for metals, but the selectivity and affinity are low. The membrane potential, which is negative on the inside of the plasma membrane, provides a strong driving force for the uptake of cations (Sanità di Toppi and Gabbrielli 1999, Benavides *et al*. 2005). So toxic non-essential metals compete with the transport systems operating in micronutrient uptake, to gain access to the plant cell. In particular, the uptake of Cd ions occurs via the same

transmembrane carriers used to take up Ca, Fe, Mg, Cu and Zn (Perfus-Barbeoch *et al*. 2002, Clemens 2006).

Passage of the plasma membrane by metals is enhanced by intracellular binding and sequestration. Once inside the cell, metals are bound to chelators and chaperones. Chelators sequester free metal ions in the cytosol or in subcellular compartments, and in this way contribute to metal detoxification. Examples are phytochelatins (PCs), metallothioneins (MTs), organic acids and amino acids (Clemens 2001, Haydon and Cobbett 2007). The major site of metal sequestration in roots seems to be the vacuole. Metal chaperones bind and deliver metal ions to organelles and metal-requiring proteins (Grotz and Guerinot 2006, Puig *et al*. 2007). The activities of metal-sequestering pathways in root cells are crucial in determining the rate of metal translocation to the aerial parts. Copper ions are retained in the roots, whereas in case of Cd exposure, a certain amount of the Cd taken up by the roots is translocated to the shoots (Benavides *et al*. 2005).

1.3.2. Physiological effects of Cd and Cu

The main and visible symptoms of metal toxicity are leaf chlorosis and growth inhibition, caused by the interference of the metal with photosynthesis, mineral nutrition and the water balance (Dalcorso *et al*. 2008, Cuypers *et al*. 2009, Yruela 2009).

These metal-induced effects can be caused by a direct interaction of the metal with thiol-, histidyl- and carboxyl-groups of proteins targeting structural, catalytic and transport sites of the cell, thereby inhibiting their function (Figure 1.1.) (Sharma and Dietz 2009). Cadmium can bind strongly to thiol groups of cysteine-rich proteins, like PCs and MTs, leading to chelation of free metal ions but also causing exhaustion of the glutathione (GSH) pool, which is also used for the detoxification of reactive oxygen species (ROS). Cadmium also shows a high affinity for sulphur and nitrogen donors, interfering with the sulphur and nitrogen metabolism (Dalcorso *et al*. 2008).

Metals can also inhibit the function of proteins by displacement of essential cations from specific binding sites (Figure 1.1.) (Sharma and Dietz 2009). Examples are the replacement of Ca by Cd in the photosystem II reaction centre, causing inhibition of photosystem II activity, or Cd entering the guard cells

through voltage-dependent Ca channels, mimicking Ca activity and initiating stomatal closure (Dalcorso *et al*. 2008). Copper can inhibit the photosynthesis by substitution of the central Mg ion in chlorophyll pigments or by reducing the chlorophyll content as a consequence of Cu-induced Fe deficiency (Yruela 2009).

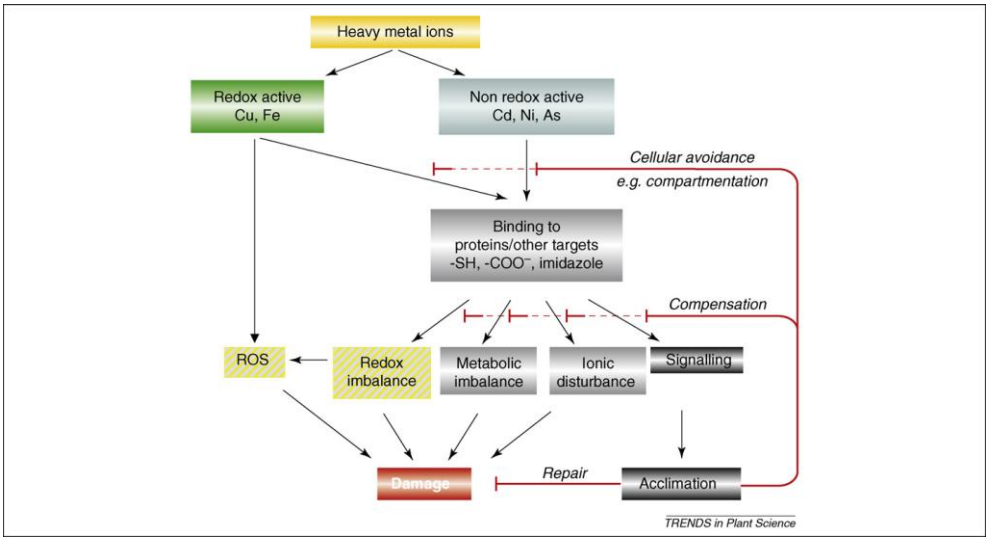


Figure 1.1.: *Mechanisms of metal-induced damage in plants (source: Sharma and Dietz 2009). Metals bind to functional groups of proteins or compete for essential cations in the catalytic domain of molecules, thereby altering target protein functions leading to changes in cell metabolism or signalling events inducing acclimation. In addition, redox-active metals, like Cu, generate directly ROS which can cause damage to macromolecules or membranes.*

Finally, metals can stimulate the production of ROS leading to oxidative stress (Figure 1.1.) (Sharma and Dietz 2009). Copper is a redox-active metal that can produce ROS directly via Fenton and Haber-Weiss reactions (Kehrer 2000). In this way, Cu causes lipid peroxidation, decreases of lipid content and changes in fatty acid composition of membranes. When this happens in the tylakoid membranes, photosystem activity is seriously impaired (Yruela 2009). Cadmium on the other hand, is not redox-active and causes oxidative stress via indirect mechanisms, like interactions with enzymes of the antioxidative defence system (Gratão *et al*. 2005, Cuypers *et al*. 2010).

1.4. Oxidative stress

At the cellular level, Cd and Cu both have the ability to induce oxidative stress (Smeets *et al*. 2009, Cuypers *et al*. 2011a). Oxidative stress is a disturbance in the cellular redox status in favour of the pro-oxidants and is often seen in stress conditions. However ROS do not act always as negative regulatory molecules in the cell. They are also produced during normal cell metabolism. In these conditions, an excess of ROS will be quenched by the antioxidative defence system of the cell, consisting of enzymes and metabolites (Mittler *et al*. 2004, Cuypers *et al*. 2011b).

1.4.1. ROS-production

Molecular oxygen ($^3O_2$) can easily be activated to toxic reactive species such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). Oxygen in its ground state ($^3O_2$) is a free radical, having two unpaired electrons with parallel spins. Singlet oxygen is generated by an input of energy that rearranges these electrons in such a way that spin restriction is alleviated. By removal of the spin restriction, the oxidizing ability of oxygen is greatly increased. Singlet oxygen can directly oxidize proteins, DNA and lipids (Edreva 2005, Halliwell 2006).

Oxygen can also be activated by stepwise monovalent reduction. The superoxide radical anion is produced by supplying O_2 with a single electron. It's a moderately reactive, short-lived ROS that cannot cross biological membranes and is rapidly dismutated to H_2O_2. Addition of another electron to $O_2^{\bullet -}$ gives rise to the peroxide ion (O_2^{2-}). In biology, the two-electron reduction product of O_2 is H_2O_2, which is not a free radical because all of its electrons are paired. Because it is small and uncharged, H_2O_2 can easily pass the membrane (Edreva 2005, Halliwell 2006). However, together with $O_2^{\bullet -}$, H_2O_2 can give rise to the highly reactive $^{\bullet}OH$ via the Fenton and Haber-Weiss reactions (Figure 1.2.) (Kehrer 2000). The hydroxyl radical can irreversibly damage cellular components, like DNA, proteins and other small molecules, and has the ability to initiate lipid peroxidation.

Oxidation of organic substrates may proceed by two possible reactions: addition of $^{\bullet}OH$ to an organic molecule or abstraction of a hydrogen atom from it. In the

addition reaction, the $^{\bullet}OH$ is added to an organic substrate forming a hydroxylated product (Arora *et al*. 2002).

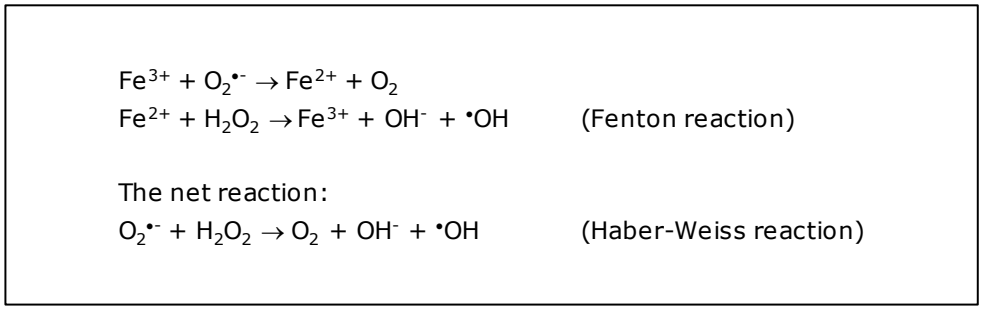


Figure 1.2.: *Fenton and Haber-Weiss reactions.*

In the abstraction reaction, $^{\bullet}OH$ oxidizes an organic substrate by forming water and an organic radical (Figure 1.3.: initiation step). This organic radical has a single unpaired electron and can react with O_2 leading to the formation of a peroxy-radical. The peroxy-radical can abstract hydrogen from another organic molecule leading to the formation of a second organic radical (Figure 1.3.: propagation step) (Arora *et al*. 2002).

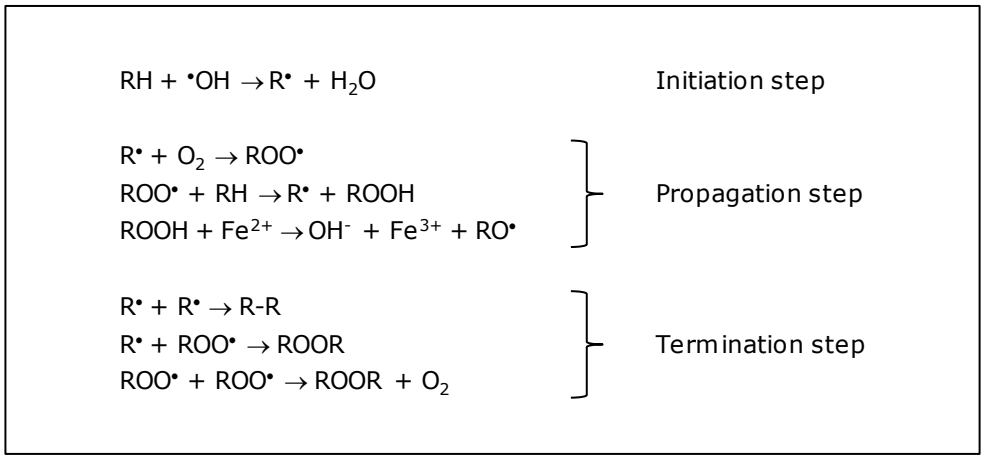


Figure 1.3.: *The chain reactions of lipid peroxidation.*

This chain reaction is more damaging than any other reaction catalysed by ROS and is in cell membranes best demonstrated by lipid peroxidation of linolenic acid. The lipid peroxides (ROOH) are unstable in the presence of Fe^{2+} or other

reduced metal ions (such as Cu^{+}), as they participate in a Fenton reaction leading to the formation of a reactive alkoxy radical (Figure 1.3.: propagation step). This alkoxy radical is as damaging as $^{\bullet}OH$ and can start a cascade of oxidative reactions. In the termination step (Figure 1.3.), products of the previous reactions can react with each other and form fatty acid or peroxide bridged dimers (Arora *et al*. 2002).

1.4.2. Mechanisms of ROS production

In plant cells, ROS can be produced during normal cell metabolism via a number of routes since most cellular compartments have the potential to become a source of ROS (Figure 1.4.) (Bhattacharjee 2005, De Gara *et al*. 2010). In the chloroplast, $O_2^{\bullet-}$ is produced in photosystem I (PSI) as a result of electron spilling from reduced ferredoxin to O_2 during the Mehler reaction and gives rise to H_2O_2 by either spontaneously or superoxide dismutase (SOD)-catalyzed dismutation. The mitochondrial electron transport system is also a potential source of ROS, including $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$. Direct reduction of O_2 to $O_2^{\bullet-}$ takes place in the flavoprotein region of NADH dehydrogenase segment of the respiratory chain. Hydrogen peroxide is also generated in the peroxisomes during the oxidation of glycolate in the C_2 pathway or during the β-oxidation of fatty acids. Other important sources of ROS in plants are detoxification reactions catalyzed by cytochrome P_{450} in the cytosol and endoplasmatic reticulum (ER) (Foyer and Noctor 2009). Furthermore, cell wall peroxidases and oxidases are able to produce ROS. These enzymes use H_2O_2 to catalyze the oxidation of NADH to NAD^{+}, which in turn reduces O_2 to $O_2^{\bullet-}$. Superoxide consequently dismutates to produce H_2O_2 and O_2 (Bolwell et al. 2002). Reactive oxygen species are also generated in plants at the plasma membrane level (lipoxygenases) or extracellularly in the apoplast via NADPH oxidases (respiratory burst oxidase homologs) (Torres and Dangl 2005, Skorzynska-Polit *et al*. 2006).

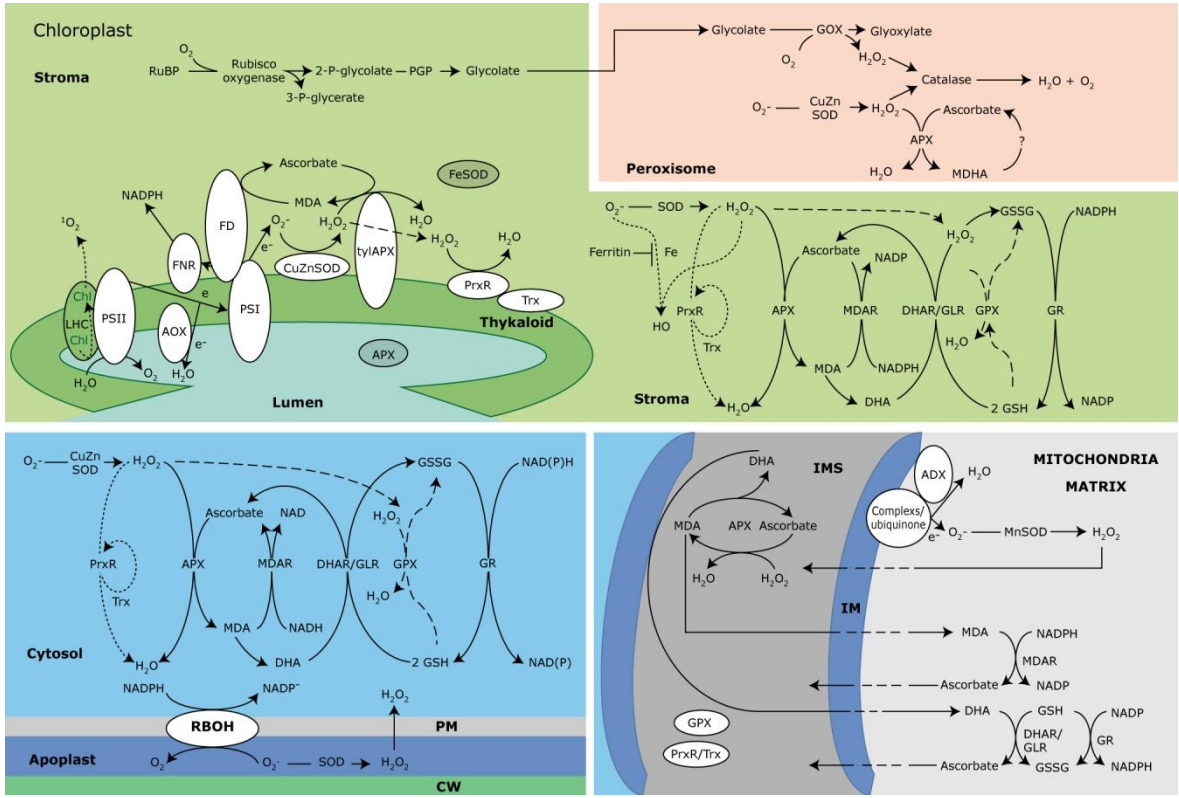


Figure 1.4.: *Localization of ROS generation and scavenging pathways in plant cells (source: Miller et al. 2010). Membrane-bound enzymes are depicted in white, GPX pathways are indicated by dashed lines and PrxR pathways are indicated by dotted lines in the stroma and cytosol. AOX, alternative oxidase; APX, ascorbate peroxidase; CAT, catalase; Chl, chlorophyll; CuZnSOD, Cu/Zn superoxide dismutase ; CW, cell wall; DHA, dehydroascrobate; DHAR, DHA reductase; FD, ferredoxin; FNR, ferredoxin NADPH reductase; GLR, glutaredoxin; GR, glutathione reductase; GOX, glycolate oxidase; GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; IM, inner membrane; IMS, IM space; LHC, light-harvesting complex; MDA, monodehydroascorbate; MDAR, MDA reductase; PGP, phosphoglycolate phosphatase; PM, plasma membrane; PrxR, peroxiredoxin; PSI, photosystem I; PSII, photosystem II; RBOH, respiratory burst oxidase homolog; RuBP, ribulose-1,5-bisphosphate; Rubisco, RuBP carboxylase oxygenase; Trx, thioredoxin.*

1.4.3. ROS-scavenging

The steady-state level of ROS has to be kept under tight control because an over-accumulation can result in cell death. Therefore, the cell contains an efficient ROS-scavenging system consisting of antioxidative enzymes and metabolites like ascorbate (AsA), glutathione (GSH), α-tocopherol, flavonoids and carotenoids (Figure 1.4.) (Mittler 2002). Within the first category, the SODs constitute the first line of defence against ROS. They are responsible for the scavenging of $O_2^{\bullet-}$ and are located in all parts of the cell: FeSOD in the chloroplast, MnSOD in the mitochondria and peroxisomes, and Cu/ZnSOD in the cytosol, the chloroplast and extracellular space. Hydrogen peroxide produced by this dismutation can be detoxified by catalase (CAT) in the peroxisomes or by ascorbate peroxidase (APX) and glutathione peroxidase (GPX) located in the cytosol, chloroplasts, mitochondria, apoplast and peroxisomes.

The removal of H_2O_2 by APX uses AsA in the AsA-GSH cycle, which is oxidized to monodehydroascorbate (MDA). Regeneration of MDA to AsA occurs via monodehydroascorbate reductase (MDAR) using NAD(P)H as a reducing equivalent. Monodehydroascorbate can also spontaneously dismutate to dehydroascorbate (DHA), which is reduced to AsA by dehydroascorbate reductase (DHAR) oxidizing GSH at the same moment. Reduction of oxidized GSH (GSSG) is performed by glutathione reductase (GR) using NAD(P)H. Detoxification of H_2O_2 by GPX also oxidizes GSH. Beside APX and GPX, other types of peroxidases exist in plants. Among these, peroxiredoxins (PrxR) have an important function alongside APX in H_2O_2 removal in the chloroplast and cytosol. Other peroxidases may be more important in the metabolism of organic peroxides (some thiol-based peroxidases) or oxidation of organic compounds with possibly incidental H_2O_2 removal (Mittler *et al*. 2004, Gratão *et al*. 2005, Foyer and Noctor 2009, Jozefczak *et al.* 2012).

1.4.4. ROS-regulation

Although ROS can react with biomolecules, which can get irreversibly damaged leading to necrosis and cell death, they can also act as signals in diverse biological processes in plants. In this way, they can influence signal transduction pathways and gene expression, suggesting that cells have evolved strategies to

utilize ROS as signals for controlling various biological programs. Reactive oxygen species are suited to act as signalling molecules since they are small and can diffuse over short distances. Among the different ROS, only H_2O_2 can cross plant membranes and therefore directly function in cell-to-cell signalling (Mittler *et al*. 2004, Pitzschke *et al*. 2006). In plant cells, H_2O_2 acts as a signal molecule involved in acclimatory signalling triggering tolerance to various abiotic and biotic stresses, like metal stress, ultraviolet radiation, salt stress, drought stress, light stress, temperature stress and in plant-pathogen interactions (Vandenabeele *et al*. 2004, Kalbina and Strid 2006, Suzuki and Mittler 2006, Torres *et al*. 2006, Miller *et al*. 2010, Opdenakker *et al*. 2012: chapter 4). Hydrogen peroxide has also been shown to act as a key regulator in a broad range of physiological processes, such as senescence, stomatal movement, programmed cell death, and growth and development (Foreman *et al*. 2003, Bright *et al*. 2006, De Pinto *et al*. 2012, Rogers 2012). Downstream signalling events associated with ROS sensing involve Ca and Ca-binding proteins, such as calmodulin; the activation of G-proteins; the activation of phospholipid signalling, which results in the accumulation of phosphatidic acid; and/or activation of mitogen-activated protein kinase (MAPK) pathways (Mittler *et al*. 2004, Apel and Hirt 2004). Hydrogen peroxide can modulate the activity and regulation of different components of MAPK cascades, such as protein phosphatases, protein kinases and transcription factors (Miller *et al*. 2008, Quan *et al*. 2008).

1.5 MAPK signalling

1.5.1 MAPKinases: nomenclature and classification

MAPK cascades are important signalling modules that convert signals generated from the receptors/sensors to cellular responses. They are composed of three protein kinase modules: MAPKK kinases (MAPKKK), MAPK kinases (MAPKK or MKK) and MAP kinases (MAPK or MPK). When MAPKKKs, serine/threonine kinases, are activated, they can phosphorylate MAPKKs via serine/threonine residues in the S/T-X_5-S/T motif. MAPKKs are dual-specificity kinases that activate MAPKs through phosphorylation of both tyrosine and serine/threonine residues in the TXY motif. MAPKs are kinases that phosphorylate a variety of

substrates, including transcription factors, transcription regulators, splicing factors and other protein kinases (Mishra *et al*. 2006).

In *Arabidopsis*, 60 MAPKKKs, 10 MAPKKs and 20 MAPKs have been identified (Table 1.1.). The MAPKKKs form the largest and most heterogeneous group of MAPK components. They can be divided in two large subgroups: the MEKK-type, for which MAPKKK function is provided, and the Raf-like kinases, for which MAPKKK function is not yet known. In *Arabidopsis*, examples of MEKK-like kinases are ANP1/2/3 and MEKK1. CTR1 and EDR1 belong to the Raf-like kinases (Ichimura *et al*. 2002).

MAPKKs (or MKKs) are the smallest group, only half as many as there are MAPKs. MAPKKs are probably able to activate multiple MAPKs, which suggests interplay between different signal transduction pathways occurring at this level. Plant MAPKKs are subdivided in four groups (A-D) based on sequence alignment. Group A MAPKKs seem to be involved in multiple abiotic stresses and cell division and are responsive to pathogens. MAPKKs belonging to group C are abiotic stress-responsive and function upstream of group A MAPKs, which play a role in environmental and hormonal responses (Ichimura *et al*. 2002).

Arabidopsis MAPKs (or MPKs) can be classified into two subtypes: those containing a TEY amino acid motif and those containing a TDY amino acid motif. The TEY subtype consists of three groups: A, B and C. MAPKs of group A are particularly involved in environmental and hormonal responses and examples of this group are MPK3 and MPK6. Group B MAPKs, to which MPK4 belongs, also play a role in environmental stress responses and seem to be involved in cell division. Little is known about group C MAPKs, but they appear to be circadian-rhythm-regulated. The TDY subtype forms group D. These MAPKs have a more extended C-terminal region in relation to groups A, B and C. On the other hand, they lack the C-terminal CD DOMAIN found in groups A, B and C, which serves as a docking site for MAPKKs, phosphatases and protein substrates. Group D MAPKs are shown to be induced by blast fungus and wounding (Ichimura *et al*. 2002).

Table 1.1.: *Classification of MAPKinases. In the first and second column, the different MAPKinase modules and the amino acid sequences phosphorylated by them are presented. Then MAPKKKs, MAPKKs and MAPKs are classified according to their phosphorylated amino acid sequence and placed in groups A to D based on sequence alignment. In the final 2 columns, examples of MAPKinases belonging to each group as well as the plant responses, in which they are involved, are provided. MAPkinases indicated in bold are discussed in this review.*

CLASSIFICATION OF MAPKINASES					
MAPKinase	**Amino acids phosphorylated by the kinase**	**Phosphorylated amino acid motif**	**Group**	**Examples**	**Response to/in**
MAPKKK	serine/threonine	MEKK1-type	A	**MEKK1**, MEKK2, MEKK3, MEKK4	redox control - oxidative stress abiotic stresses: drought, salt, mechanical biotic stress: bacterial pathogens hormones: salicylic acid
				ANP1, ANP2, ANP3	redox control - oxidative stress biotic stress: bacterial pathogens hormones: auxin cytokinesis
		Raf-like	B	EDR1, **CTR1**	hormones: ethylene disease resistance signaling
			C	ATN1, MRK1	unknown
MAPKK	threonine/tyrosine	S/T-XXXXX-S/T	A	**MKK1**, **MKK2**, MKK6	redox control - oxidative stress abiotic stresses: cold, salt, low humidity, mechanical biotic stresses: bacterial pathogens hormones: salicylic acid cell division
			B	**MKK3**	oxidative stress abiotic stress: mechanical biotic stress: bacterial pathogens hormones: jasmonic acid

Table 1.1.: *Cont.*

CLASSIFICATION OF MAPKINASES					
MAPKinase	**Amino acids phosphorylated by the kinase**	**Phosphorylated amino acid motif**	**Group**	**Examples**	**Response to/in**
MAPKK	threonine/tyrosine	S/T-XXXXX-S/T	C	**MKK4**, **MKK5**	redox control - oxidative stress abiotic stresses: salt biotic stresses: bacterial pathogens hormones
			D	MKK7, MKK8, **MKK9**, MKK10	oxidative stress biotic stress: bacterial pathogens
MAPK	serine/threonine/tyrosine	TEY	A	**MPK3**, **MPK6**, MPK10	redox control - oxidative stress abiotic stresses: salt, cold biotic stresses: bacterial pathogens hormones: jasmonic acid
			B	**MPK4**, MPK5, MPK11, MPK12, MPK13	redox control - oxidative stresses abiotic stresses: cold, salt, low humidity, mechanical hormones; salicylic acid cell division
			C	**MPK1**, **MPK2**, **MPK7**, MPK14	oxidative stress abiotic stress: mechanical biotic: bacterial pathogens hormones: jasmonic acid, abscisic acid circadian-rhythm-regulated
		TDY	D	**MPK8**, MPK9,MPK15/16/17/18/19/20	oxidative stress abiotic stress: mechanical biotic: blast fungus

The MAPKKKs are the most divergent group of kinases in the MAPK cascade. Therefore different MAPKKKs can initiate similar MAPK cascades, which finally activate the same downstream MAPK. This is one of the mechanisms by which different stimuli converge onto one MAPK. The sharing of a single component by different cascades also leads to interaction between different pathways (Ichimura *et al*. 2002, Jonak *et al*. 2002).

1.5.2. Regulation of MAPKinases

The outcome of a MAPK activation depends on the duration of its activation. The length of time that a MAPK remains active, depends on the upstream specific regulation mechanisms, of which scaffolding (co-localization) and attenuation through phosphatases are best known. In addition, attention is given to lipid signalling which can initiate MAPK cascades.

1.5.2.1. Scaffolding

Specificity of different MAPK cascades functioning within the same cell is conferred by docking domains of scaffold proteins, which anchor different MAPK modules in one complex (Morrison and Davis 2003). MAPK components themselves can also function as scaffolds. An example is the MAPKKK 'Oxidative stress-activated MAP triple-kinase 1' (OMTK1), discovered in *Medicago sativa*. OMTK1 is able to activate MMK3 in response to H_2O_2 but not in case of cell treatment with yeast elicitor or 1-aminocyclopropane-1-carboxylic acid (ACC). Pull-down analysis between recombinant proteins showed that OMTK1 directly interacts with MKK3 and that both kinases are found together in a protein complex *in vivo*, suggesting that OMTK1 can determine specificity through its scaffolding function (Nakagami *et al*. 2004). In *Arabidopsis*, no scaffold proteins of MAPK components with scaffold function are known to date.

1.5.2.2. Phosphatases

Negative regulation of MAPK cascades is performed by MAPK phosphatases, which dephosphorylate threonine and tyrosine residues within the activation motif of MAPKs. Three different forms of phosphatases are identified to date: protein tyrosine phosphatases (PTPs), protein serine-threonine phosphatases (PSTPs) or dual-specificity (Ser/Thr and Tyr) phosphatases (DSPs) (Bartels *et al*.

2010). In *A. thaliana*, members of all three classes are linked with MAPK inactivation, but dual-specificity MAPK phosphatases are the most important group because full inactivation of MAPKs requires dephosphorylation of both residues. The *Arabidopsis* genome encodes five possible candidate dual-specificity MAPK phosphatases (MKP1, MKP2, DsPTP1, PHS1 and IBR5) (Bartels *et al*. 2010).

MKP1 was first identified by its involvement in genotoxic stress resistance. When 5-day-old seedlings were subjected to UV-C radiation (0.5-1 kJ/m^2) or 50 mg l^{-1} methyl methane sulphonate, MKP1 was required for maintaining proper MAPK activity levels. Yeast-two-hybrid assays showed that MKP1 interacts with the stress-activated MAPKs MPK3, MPK4 and MPK6. Interaction of MKP1 with MPK6 was the most pronounced and MKP1 was reported to regulate MPK6 activity *in vivo* (Ulm *et al*. 2001, 2002). In addition, a role for MKP1 as a negative regulator of MPK3 and MPK6 activities was suggested in resistance against the bacterial pathogen *Pseudomonas syringae* and tolerance against UV-B and salt stresses (Ulm *et al*. 2002, Anderson *et al*. 2011, Besteiro *et al*. 2011). Lee *et al*. (2008) demonstrated that MKP1 activity increased after binding to calmodulin, indicating that Ca- and MAPK signalling are connected via the regulation of MKP1. MKPs can act together with other protein phosphatases to control MAPK activity. The protein tyrosine phosphatase 1 (PTP1), which was also shown to interact with MPK6, acts together with MKP1 to repress the biosynthesis of salicylic acid (SA) and camalexin, and pathogenesis-related gene expression, which makes plants more vulnerable to infection with *Pseudomonas syringae* (Bartels *et al*. 2009).

Another MKP, MKP2, is also reported to bind and dephosphorylate MPK3 and MPK6 *in vitro* as well as *in vivo* (Lee and Ellis 2007, Lumbreras *et al*. 2010). After acute exposure to 500 ppb ozone, MKP2 acts as a positive regulator of the cellular redox status by repressing the activity of MPK3/6 (Lee and Ellis 2007). In case of plant-pathogen interactions, MKP2 exerts differential and specific functions depending on the invading pathogen and is required for maintaining adequate levels of MPK3/6 activation. The appearance of bacterial wilting symptoms was delayed with one day in *mkp2* homozygous knockout plants infected with *Ralstonia solanacearum*. In contrast, *mkp2* knockout plants infected with *Botrytis cinerea* showed a systemic spread of the fungus

throughout the whole plant after 15 days of inoculation, whereas in wild type plants lesions were local and restricted to the inoculated leaves (Lumbreras *et al*. 2010).

1.5.2.3. Lipid signalling

Besides the regulation of MAPKs by protein phosphatases, MAPK cascades can also be regulated via lipid signalling. Plasma membrane-associated phospholipase D (PLD) enzymes release phosphatidic acid (PA) from phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol. PA has been implicated as a secondary messenger in many different stress responses, such as the production of ROS (Bargmann and Munnik 2006).

In *Arabidopsis*, Yu *et al*. (2010) showed that PLD-derived PA binds to MPK6, leading to its activation during salt stress. Activated MPK6 is responsible for the phosphorylation of the Na/H antiporter SOS1, which reduces Na accumulation in *Arabidopsis* leaves. In *Arabidopsis*, Anthony *et al*. (2004) reported that binding of PA to '3-phosphinositide-dependent protein kinase 1' (PDK1) stimulates phosphorylation and activation of the serine/threonine protein kinase 'Oxidative-signal inducible 1' (OXI1). Activity of OXI1 was induced within 30 min after treatment of *Arabidopsis* plants with plant growth factors, like auxin and cytokinin, suggesting a role for OXI1 in plant growth and cell division. Rentel *et al*. (2004) revealed that OXI1 is involved in H_2O_2-dependent activation of MPK3/6 in ROS-dependent processes such as root-hair elongation and basal resistance to the fungal pathogen *Peronospora parasitica*. In addition, PA-stimulated activation of the PDK1/OXI1/MPK6 pathway was shown to promote plant growth in *Arabidopsis* seedlings after co-cultivation with the endophytic fungus *Piriformospora indica* (Camehl *et al*. 2011). Activation of MAPKs by OXI1 is mediated by serine/threonine protein kinases of the Pto-interacting 1 (PTI1) like family or NDP kinase 2 (NDPK2) (Anthony *et al*. 2006, Forzani *et al*. 2011, Moon *et al*. 2003). However, further research is required to unravel the OXI1 – MAPK cascade.

1.5.3. Role of MAPK cascades in stress response signalling

MAPK cascades are involved in normal cell metabolism like physiological, developmental and hormonal responses (Mishra *et al*. 2006, Zhang *et al*. 2006,

Pitzschke and Hirt 2009). However multiple studies have shown that MAPK cascades play important roles in plant responses to biotic and abiotic stresses, such as pathogen infection, wounding, low temperature, drought, hyper- and hypo-osmolarity, high salininty, mechanical stress, metals and ROS (Zhang and Klessig 2001, Colcombet and Hirt 2008, Pitzschke and Hirt 2009).

1.5.3.1. MAPK cascades are involved in metal stress

Several authors reported the involvement of MAPK signalling in metal stress for different plant species (Table 1.2.). In general, mRNA as well as activity levels are increased quickly after metal exposure, ranging from 5 min to 1h, and activation of MAPKs is transient. In *Arabidopsis*, it is proven that MPK3 and MPK6 are activated in response to short-term exposure (less than 1h) to $CdCl_2$ concentrations as low as 1 µM, via the accumulation of ROS (Liu *et al*. 2010). However, so far, evidence for the involvement of a complete MAPK cascade pathway in metal stress responses is rather scarce in plants.

Rao *et al*. (2011) predicted a possible MAPK cascade in rice namely OsMKK4/OSMPK3. Two weeks old rice plants exposed to 50 µM arsenite showed increased transcript levels of *OsMPK3* in leaves and roots already after 30 min of exposure. These results were confirmed at the protein level: activity of OsMPK3 was elevated within 3h. Gene expression levels of *OsMKK4* were also elevated in leaves and roots after 3h exposure to arsenite. In *Medicago sativa* roots, transient activation of MAPKs (SIMK, SAMK, MKK2 and MMK3) was rapidly induced (less than 10 min) after exposure to 100 µM $CuCl_2$, whereas treatment with 100 µM $CdCl_2$ delayed this profile. In addition, transient expression assays in *Arabidopsis* protoplasts with HA-tagged SIMK, SAMK, MMK2 and MKK3, and a myc-tagged MAPKK (SIMKK), showed that SIMKK specifically activated SIMK and SAMK after exposure to 100 µM $CuCl_2$ (Jonak *et al*. 2004). Opdenakker *et al*. (2012; chapter 4) showed that 24h exposure of *Arabidopsis thaliana* seedlings to environmental realistic concentrations of Cu and Cd increased transcript levels of MAPKinases in a time-dependent manner. Already 2h after exposure to 2 µM Cu, gene expression of *OXI1*, the MAPKKK 'Arabidopsis NPK1-like protein kinase 1' (*ANP1*) and the MAPKs *MPK3* and *MPK6* was affected in roots and leaves of *Arabidopsis* plants. After exposure to 5 µM Cd, no changes in gene expression of these enzymes were observed before 24h. These changes in

gene expression seemed to be related to the production of H_2O_2 by these metals, either directly and fast by Cu (Fenton-HaberWeiss reactions) or indirectly and delayed by Cd (e.g. via NADPH oxidases).

Activation of ANP1 and OXI1 by H_2O_2 and induction of a phosphorylation cascade involving MPK3 and MPK6 has been reported before in *Arabidopsis* leaf cells and whole plants (Kovtun *et al*. 2000, Rentel *et al*. 2004). Application of 200 µM H_2O_2 to *Arabidopsis* protoplasts, increased the activity of ANP1, MPK3 and MPK6 within 10 min. Co-transfection of protoplasts with ANP1 and MPK3 or MPK6 revealed that ANP1 could further enhance the activity of MPK3 and MPK6 after H_2O_2 treatment (Kovtun *et al*. 2000). Rentel *et al*. (2004) showed that gene expression of *OXI1* was already enhanced after 30 min in 7-day-old seedlings treated with 10 mM H_2O_2 and *oxi1* knockout mutants failed to activate MPK3 and MPK6 after treatment with H_2O_2. Additionally, a toxicity test based on primary root elongation showed that *oxi1* and *mpk6* knock-outs were more tolerant to excess Cu, but not Cd, suggesting that OXI1 and MPK6 play important roles in the observed stress response following Cu exposure (Opdenakker *et al*., unpublished data).

However, knowledge about the downstream signalling targets of MAPKs is rather scarce under metal stress. Roelofs *et al*. (2008) compared known signalling pathways induced by metals stress as well as by other abiotic stresses (cold, heat, salt, drought) between soil invertebrates and plants. They showed that all abiotic stresses switched on more than one stress-responsive pathway, seen in the overlap of transcription factors used by each stressor, and they speculated that bZIP, MYB and MYC transcription factors could be downstream targets of MAPK signalling in plant metal stress.

Interplay between the MAPK pathways activated by metal stress and the ones used by other stresses probably exists because ROS generation, which is known to induce MAPK signalling, is common to other abiotic and biotic stress responses (Figure 1.5.).

Table 1.2.: *Induction of MAPKinases under metal stress. MAPK cascade modules affected by exposure to metals are categorized based upon plant species and type of kinase. Exposure to metals influences MAPKinase mRNA levels as well as the activity at the protein level.*

METAL-INDUCED MAPKINASES							
Plant	**Component of MAPK cascade**		**Metal**	**Concentration**	**Exposure Time**	**Observations**	**Reference**
Arabidopsis thaliana	**MAPKKK**	MEKK1	Cd	500 µM $CdCl_2$	1 - 3h	↑ mRNA levels	Suzuki *et al*. 2001
	MAPK	MPK3/MPK6	Cd	1 µM $CdCl_2$	10 min	↑ activity	Liu *et al*. 2010
			Cu/Cd	2 µM $CuSO_4$ / 5 µM $CdSO_4$	2-24h	↑ mRNA levels	Opdenakker *et al*. 2012
Brassica juncea	**MAPK**	46 kDa MAPK	As(III)	50 µM As(III)	15 min - 1h	↑ activity	Gupta *et al*. 2009
Medicago sativa	**MAPKK**	SIMKK	Cu	100 µM $CuCl_2$	30 min	induces SAMK and SIMK	Jonak *et al*. 2004
	MAPK	SAMK	Cu/Cd	100 µM $CuCl_2$ / $CdCl_2$	10 min - 1h (Cu) / 30 min - 3h (Cd)	↑ activity	Jonak *et al*. 2004
		SIMK	Cu/Cd	100 µM $CuCl_2$ / $CdCl_2$	5 min - 6h (Cu) / 10 min - 6h (Cd)	↑ activity	Jonak *et al*. 2004
		MMK2	Cu/Cd	100 µM $CuCl_2$ / $CdCl_2$	10 min - 1h (Cu) / 10 min - 3h (Cd)	↑ activity	Jonak *et al*. 2004
		MMK3	Cu/Cd	100 µM $CuCl_2$ / $CdCl_2$	10 min - 1h (Cu) / 10 min - 1h (Cd)	↑ activity	Jonak *et al*. 2004
Oryza sativa	**MAPKK**	OsMKK4	As	50 µM As(III)	3 - 9h	↑ mRNA levels	Rao *et al*. 2011
	MAPK	OsMSRMK2	Cu/Cd/Hg	100 µM $CuSO_4$/$CdCl_2$/$HgClO_3$	30 min	↑ mRNA levels	Agrawal *et al*. 2002
		OsMSRMK3	Cu/Cd/Hg	100 µM $CuSO_4$/$CdCl_2$/$HgClO_3$	15 min - 2h	↑ mRNA levels	Agrawal *et al*. 2003
		OsWJUMK	Cu/Cd/Hg	100 µM $CuSO_4$/$CdCl_2$/$HgClO_3$	15 min - 2h	↑ mRNA levels	Agrawal *et al*. 2003
		OsMPK2	Cd	400 µM $CdCl_2$	3 - 12h	↑ mRNA levels	Yeh *et al*. 2004
			Cu	100 µM $CuCl_2$	3 - 12h	↑ mRNA levels	Yeh *et al*. 2003, Hung *et al*. 2005
		OsMPK3	Cu/Cd	50 µM $CuCl_2$ / 100 µM $CdCl_2$	1h	↑ activity	Yeh *et al*. 2007
			As	50 µM As(III)	30 min - 9h	↑ mRNA and activity levels	Rao *et al*. 2011
		OsMPK4	As	50 µM As(III)	30 min - 9h	↑ mRNA and activity levels	Rao *et al*. 2011
		OsMPK6	Cu/Cd	50 µM $CuCl_2$ / 100 µM $CdCl_2$	1h	↑ activity	Yeh *et al*. 2007
		40 kDa MAPK	Zn	1 mM $ZnCl_2$	15 min - 8h	↑ activity	Lin *et al*. 2005
			Pb	10 mM $Pb(NO_3)_2$	30 min - 8h	↑ activity	Huang and Huang 2008
		42 kDa MAPK	Zn	1 mM $ZnCl_2$	15 min - 8h	↑ activity	Lin *et al*. 2005
			I	500 µM $FeSO_4$	15 - 30 min	↑ activity	Tsai and Huang 2006
			Pb	10 mM $Pb(NO_3)_2$	15 min - 8h	↑ activity	Huang and Huang 2008
Zea mays	**MAPK**	ZmMPK3	Cd	500 $CdCl_2$	30 min - 1h	↑ mRNA levels	Wang *et al*. 2010
		ZmMPK5	Cr(VI)	250 µM Cr(VI)	30 min	↑ activity	Ding *et al*. 2009

1.5.3.2. Comparison to MAPK pathways involved in other stress responses

In *Arabidopsis* protoplasts, Kovtun *et al*. (2000) showed that the MAPKKK ANP1, induced by H_2O_2, activated the downstream MAPKs MPK3/6. The MAPKKs involved in the activation of MPK3/6 by ANP1 could be MKK4 and MKK5. Ren *et al*. (2002) reported that transgenic *Arabidopsis* plants, expressing *MKK4* and *MKK5* under the control of a steroid-inducible promoter, were able to activate MPK3/6, resulting in cell death. The protein kinase OXI1 is, as already mentioned above, involved in H_2O_2-dependent activation of MPK3/6 (Rentel *et al*. 2004). Moreover, *oxi1* knockout mutants showed defects in ROS-dependent developmental processes such as root-hair elongation, and in ROS-dependent basal resistance to the fungal pathogen *Peronospora parasitica*. In what way OXI1 activates MPK3 and MPK6 remains to be addressed, although it is suggested that this activation may be modulated by NDPK2 (Moon *et al*. 2003, Colcombet and Hirt 2008). Exposure of 2-week-old *Arabidopsis* plants to 4 mM H_2O_2 strongly increased gene expression of *NDPK2* within 30 min and up to 12h, suggesting that NDPK2 functions in ROS homeostasis. Furthermore, overexpression of *NDPK2* resulted in lower levels of ROS as compared to wild type plants and conferred enhanced tolerance to environmental stresses that induce ROS generation, such as freezing during 1h or exposure to 50 mM NaCl for 3 weeks. Specific interaction between NDPK2 and MPK3/6 was discovered using yeast two-hybrid and *in vitro* protein pull-down assays. NDPK2 was also shown to enhance the myelin basic protein phosphorylation activity of MPK3 *in vitro* (Moon *et al*. 2003).

Nakagami *et al*. (2006) reported on another *Arabidopsis* MAPKKK, MEKK1, which is also regulated by H_2O_2 and was found to activate the MAPK MPK4 in response to treatment of *Arabidopsis* protoplasts with 2 mM H_2O_2 during 5 min. Ten-day-old *mekk1* knockout plants showed a deregulated expression of genes involved in cellular redox control, like glutathione S-transferases, NADPH oxidases and ascorbate peroxidases, and accumulated ROS, suggesting that MEKK1 is necessary for normal redox homeostasis of the cell. The MAPK cascade MEKK1-MKK1/MKK2-MPK4 was earlier identified using yeast two-hybrid and complementation analysis of yeast mutants (Ichimura *et al*. 1998, Mizoguchi *et al*. 1998). Later, it was shown that MKK1 phosphorylates MPK4 *in vitro* as well as *in vivo* and that this cascade is operating in different environmental stresses

such as low temperature, low humidity, hyper-osmolarity and mechanical stress (Ichimura *et al*. 2000, Huang *et al*. 2000, Matsuoka *et al*. 2002).

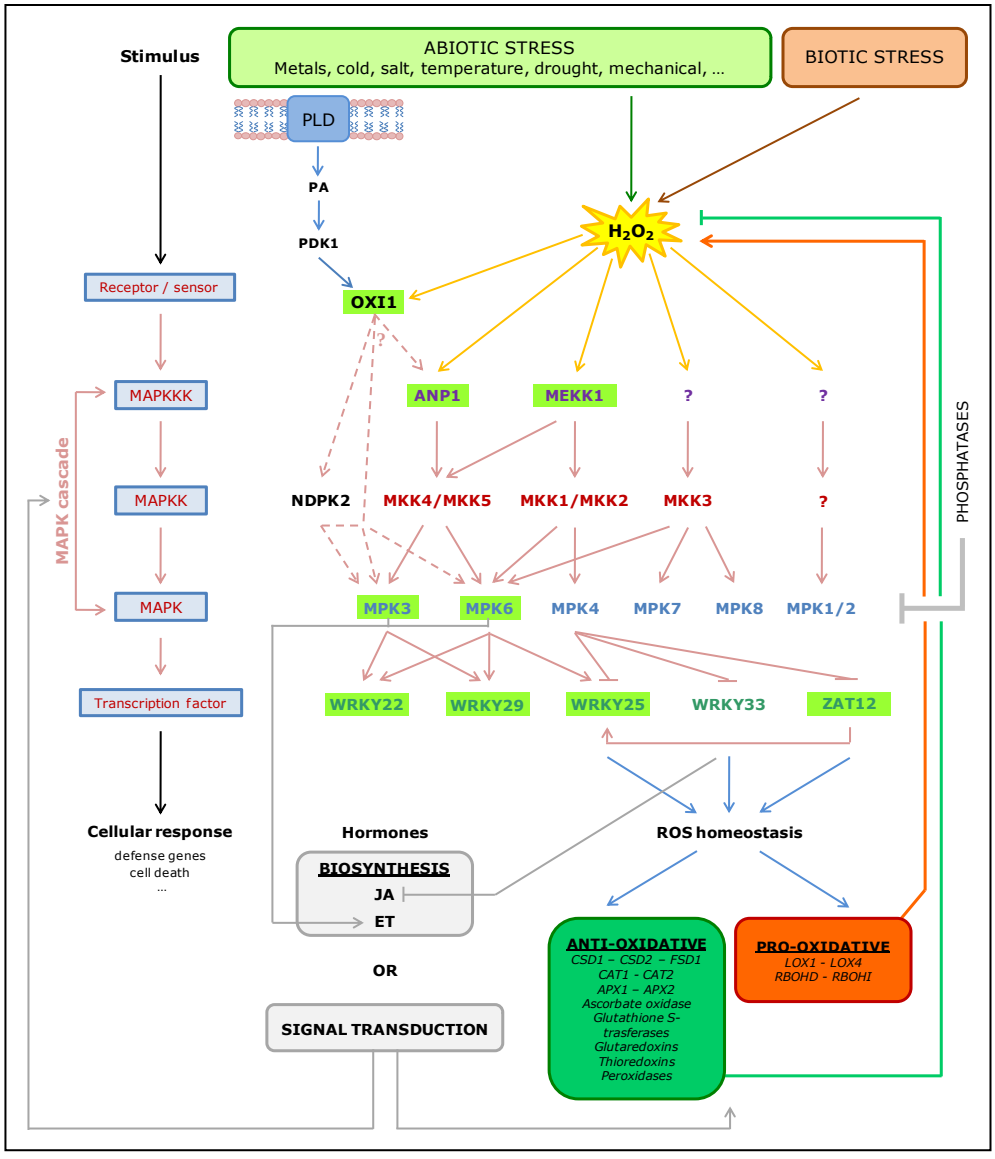


Figure 1.5.: *Overview of the different MAPK cascades known to function in stress responses as well as their regulation and possible downstream outcomes (signalling components involved in metal stress are highlighted in green). Production of ROS is a common phenomenon between different biotic and abiotic stresses. In particular H_2O_2 can act as an intra- and intercellular signalling molecule activating MAPK cascades. Via lipid signalling or phosphorylation of OXI1, ROS production can be linked to MAPK activation. MAPK signalling pathways induce gene expression of stress-responsive genes through the activation of transcription factors. On one hand, MAPK cascades influence the cellular*

redox status by activating gene expression of antioxidative or pro-oxidative enzymes. In this way, MAPK signalling can attenuate or amplify the initial ROS signal. On the other hand, MAPKs interfere with hormone signalling and biosynthesis leading to activation of downstream stress responses. Inactivation of MAPK pathways occurs through phosphatases, which dephosphorylate MAPKs, resulting in the disruption of the MAPK signal and are therefore important in the regulation of downstream responses.

A microarray study with 14-day-old *mekk1*, *mkk1/2* and *mpk4* knockout *Arabidopsis* plants performed by Pitzschke *et al*. (2009) showed that the MEKK1-MKK1/2-MPK4 cascade is a key regulator of ROS- and SA-initiated stress signalling. However, they also suggest that MEKK1 can activate another pathway, independent of MKK1/2 and MPK4, and that MKK1/2 is not only regulating MPK4, but most likely also other MAPKs like MPK3/6. Asai *et al*. (2002) showed that in protoplasts treated with 100 mM of a bacterial flagellin peptide (flg22), MEKK1 activated MPK3/6 through MKK4/MKK5, leading to the expression of early-defence response genes. Activation of the MEKK1-signalling cascade by flg22 is mediated by receptor-like kinases (RLKs), which are also reported to be regulated by Cd stress (Ding *et al*. 2011). Recently, MKK4 was also identified to be involved in abiotic salt stress responses, as a regulator of MPK3 activity (Kim *et al*. 2011). In contrast, studies performed with *mekk1* knockout plants instead of the protoplast system used by Asai *et al*. (2002) reported that 14-day-old *mekk1* knockout plants did activate MPK3 and MPK6 activity within 10 min after treatment with flg22, but failed to induce MPK4 activity. These results indicate that probably more alternative pathways exist to activate MPK3/6 in the absence of MEKK1 (Suarez-Rodriguez *et al*. 2007). MKK2, activated by MEKK1, was observed to directly target MPK4 and MPK6 in cold and salt stress (Teige *et al*. 2004). Plants overexpressing *MKK2* exhibited constitutive MPK4 and MPK6 activity, showing increased freezing and salt tolerance while *mkk2* knockout plants were impaired in MPK4 and MPK6 activation, showing hypersensitivity to salt and cold stress. These studies demonstrate that MEKK1 can integrate different stress signals and ensures stress-specific responses by activating different downstream MAPKs.

Recently, another MAPK component, able to integrate different stress signals, was identified. MKK3 acts as upstream activator of MPK7, which induces target genes such as *PR1* in the defence response against *Pseudomonas syringae.* In *Arabidopsis* protoplasts, transiently expressing *MKK3* and *MPK7*, MKK3-mediated activity of MPK7 was only induced after application of 4 mM H_2O_2 during 5 or 15

min, whereas treatment with 1 µM flg22 had no effect on the MPK7 activity. These data led to the observation that the MKK3-MPK7 cascade is induced by H_2O_2-mediated inhibition of the proteasome-dependent degradation of MKK3 (Doczi *et al*. 2007). In contrast, the MKK3-MPK6 pathway functions in jasmonic acid (JA) signalling. In 2-week-old wild type plants, MPK6 activity was enhanced directly after treatment with 50 µM JA. This activity was reduced in *mkk3* knockout plants and higher in *MKK3* overexpressing plants exposed to JA. In addition, JA-dependent inhibition of root growth and induction of *PDF1.2* and *VSP2* expression was regulated by MKK3-MPK6 (Takahashi *et al*. 2007). In case of mechanical stress, MKK3 is together with Ca/CaM responsible for the full activation of MPK8, which negatively regulates the expression of *RBOHD* (NADPH-oxidase). RBOHD plays an important role in ROS generation and the ROS signal provided by RBOHD is involved in the induction of wound-inducible marker genes, such as *OXI1* and *ZAT12*. Therefore, the negative regulation of *RBOHD* by MPK8 is required for appropriate production of ROS during mechanical stress responses (Takahashi *et al*. 2011). In addition, Ortiz-Masia *et al*. (2007) showed a rapid (within 15 min) activation in 4-week-old *Arabidopsis* plants upon treatment with 5 mM H_2O_2 as well as by other stress signals such as mechanical stress or application of 50 µM JA or 100 µM abscisic acid (ABA).

1.5.4. MAPK cascades regulate stress responses by activation of gene transcription

1.5.4.1. Transcription factors

MAPK cascades have the possibility to regulate gene transcription by activation or repression of transcription factors. Popescu *et al*. (2009) used high-density *Arabidopsis* protein microarrays to identify *in vitro* novel MPK targets. They observed that the largest group of possible MPK targets identified in their screen, represent transcription factors. Coexpression of WRKY and TGA transcription factors with specific MKK/MPK modules showed that these transcription factors are also phosphorylated *in vivo*.

WRKY proteins bind to W-box DNA elements (containing a TGAC core sequence) and act both as positive and negative regulators of target gene expression.

WRKY family members are subdivided into three groups based on the number of WRKY domains and certain features of the zinc finger-like motifs (for a review, see Eulgem et al. (2000) and references therein). WRKY transcription factors are known to be involved in diverse biotic and abiotic stresses (Figure 1.5.). Short-term exposure of 3-week-old *Arabidopsis* plants to 2 µM Cu, induced gene expression of *WRKY22*, *WRKY25* and *WRKY29* already after 2h of exposure in leaves and roots. In contrast, after 24 h of exposure to 5 µM Cd only the gene expression of *WRKY25* and *WRKY29* was affected. These data suggest that these transcription factors play an essential role in regulation of the stress response upon metal exposure (Opdenakker *et al*. 2012; chapter 4). Involvement of these WRKY transcription factors was also reported in other stress conditions confirming the hypothesis that use of these transcription factors is not specific for metal stress signalling but is common between different biotic and abiotic stress responses. Investigation of transcriptome data generated from ROS-related microarray experiments, showed that induction of WRKY22 gene expression is also related to $^1O_2$, O_3 and $O_2^{\bullet-}$ production (Gadjev *et al*. 2006). More recently, *WRKY22* gene expression was reported to be induced by H_2O_2 in leaf senescence (Zhou *et al*. 2011). Transcript levels of *WRKY22* were induced 1h after application of 3% H_2O_2 or by dark treatment already after 1 day. The involvement of WRKY22 in dark-induced leaf senescence was investigated by comparing *wrky22* knockout plants and *WRKY22* overexpressing plants with wild type plants in relation to cell death, chlorophyll content and expression of senescence-associated genes (Zhou *et al*. 2011). The flagellin-induced MAPK cascade MEKK1-MKK4/MKK5-MPK3/MPK6 is known to activate WRKY22 and his close homolog WRKY29. They positively regulate gene expression of disease resistance genes to confer resistance to both bacterial and fungal pathogens (Asai *et al*. 2002).

In vitro and *in vivo* interaction studies revealed a MPK4 substrate MKS1 (MAP kinase 4 substrate 1), which functions in coupling MPK4 to WRKY25 and WRKY33. In this way, WRKY25/33 function in the regulation of pathogen defence responses by repression of SA-dependent resistance. Indeed, *Arabidopsis* plants overexpressing *WRKY25* showed increased disease symptoms together with an invasive bacterial growth after inoculation with *Pseudomonas syringae* during 3 and 4 days. This was due to reduced expression of *PR1*, a

molecular marker for SA-mediated defence signalling (Andreasson *et al*. 2005, Zheng *et al*. 2006). In addition, studies pointed out a role for WRKY25 in the modulation of gene transcription during heat and salt stress (Li *et al*. 2009, Jiang and Deyholos 2009). Transcript levels of *WRKY25* were induced within 30 min in *Arabidopsis* plants exposed to 42°C. Five-day-old *wrky25* knockout plants showed an increased inhibition of root elongation when exposed to 45°C for 4h, whereas 3-week-old *wrky25* knockout displayed greater electrolyte leakage after 4h exposure to 42°C, indicating the positive role of *WRKY25* in thermotolerance (Li *et al*. 2009). Treatment of 3-week-old *Arabidopsis* plants with 150 mM NaCl during 6 and 24h induced gene expression of *WRKY25* as well as *WRKY33* strongly. Root growth was stimulated in *WRKY25* and *WRKY33* overexpressing plants in the presence of 100 mM NaCl, suggesting the involvement of these transcription factors in tolerance against salt stress (Jiang and Deyholos 2009).

The zinc finger transcription factor (C2H2-type) *ZAT12* is also involved during metal stress responses. As was also observed for the WRKY transcription factors, transcript levels of *ZAT12* were elevated after 2h in roots of 3-week-old *Arabidopsis* plants exposed to 2 µM Cu, whereas *ZAT12* expression in 5 µM Cd-exposed roots was not increased before 24h. In leaves, gene expression of *ZAT12* was increased after 24h in both Cu- and Cd-exposed plants (Opdenakker *et al*. 2012; chapter 4). Besides its involvement in metal stress, ZAT12 was also found to respond at the transcriptional level to other abiotic and biotic stresses. A comparison of microarray profiles of 6-week-old wild type and catalase-deficient (20% residual catalase activity) *Arabidopsis* plants exposed to high light for at least 3h, identified ZAT12 as a H_2O_2-responsive transcription factor (Vandenabeele *et al*. 2004, Vanderauwera *et al*. 2005). This observation was confirmed by the results of a microarray study performed on *Arabidopsis* cell cultures exposed to 20 mM H_2O_2 during 1.5 and 3h (Desikan *et al*. 2001). Mechanically stressed leaves of 4-week-old *Arabidopsis* seedlings showed significantly increased transcript levels of *ZAT12* after 30 min. This increase in gene expression was still visible after 6h of stress (Cheong *et al*. 2002). Activation of *ZAT12* transcription was also reported to occur in 3-week-old *Arabidopsis* plants in response to cold (4°C, 2h), heat (38°C, 1h), salt (150 mM NaCl, 4h) and drought (75% relative water content) (Davletova *et al*. 2005b).

1.5.4.2. Regulation of the cellular redox status by MAPK cascades

Metal stress, as well as other biotic and abiotic stresses, is known to disrupt the cellular redox status by stimulating the production of ROS or affecting the antioxidative defence system of the cell. Signalling via MAPK cascades can influence both sides of the redox balance (Figure 1.5.).

Pitzschke *et al*. (2009) showed that the MEKK1-MKK1/MMK2-MPK4 pathway negatively controlled the gene expression of *WRKY25*, *WRKY33* and *ZAT12*. Expression of oxidative stress responsive genes like the Cu/Zn superoxide dismutase *CSD1*, the catalase *CAT2*, the NADPH oxidase *RBOHI*, and certain glutaredoxins and thioredoxins, was also altered in *mpk4* knockout plants. This suggests that the MEKK1-MMK1/MMK2-MPK4 pathway regulates ROS homeostasis via the transcription factors WRKY25/33 and ZAT12. Also in case of heat or salt stress, WRKY25 and WRKY33 were reported to influence the gene expression of ROS-responsive genes (Li *et al*. 2009, Jiang and Deyholos 2009). Exposure of 21-day-old *wrky25* knockout plants to 42°C for 30, 60 and 120 min showed lower transcript levels of the ascorbate peroxidases *APX1* and *2* as compared to wild type plants. These data indicate that WRKY25 can positively regulate the expression of two oxidative stress-responsive genes *APX1* and *APX2* (Li *et al*. 2009). Microarray studies on salt-exposed (150 mM, 6h) *wrky33* knockout plants revealed glutathione-S-transferases, class III peroxidases and the lipoxygenase *LOX1* as possible targets of WRKY33 transcriptional activity (Jiang and Deyholos 2009). Because WRKY25 and WRKY33 share very similar protein structures (Eulgem et al. 2000), it is possible that they are involved in the regulation of the same genes. For example, Li *et al*. (2011) showed that WRKY25 and WRKY33, together with WRKY26, work coordinately to induce thermotolerance in plants.

Studies with *ZAT12* overexpressing as well as knockout plants revealed that ZAT12 is responsible for the induction of oxidative stress-related transcripts, like *APX1*, *CSD1*, *CSD2*, *RBOHD*, *LOX4* and several glutathione S-transferases, while reducing transcript levels of the iron superoxide dismutase *FSD1*, an L-ascorbate oxidase, several peroxidases and glutaredoxins (Rizhsky *et al*. 2004, Davletova *et al*. 2005a, Davletova *et al*. 2005b, Vogel *et al*. 2005). These data suggest that ZAT12 is important in facilitating plants to cope with oxidative stress. ZAT12 was also identified as inducer of WRKY25 during oxidative stress, meaning that

WRKY25 acts downstream of ZAT12 to control its target genes (Rizhsky *et al*. 2004, Li *et al*. 2009). Nakagami *et al*. (2006) suggested a role for the MEKK1-MPK4 pathway in the negative regulation of ZAT12 under oxidative stress conditions. Exposure of *mekk1* and *mpk4* knockout plants to 10 mM H_2O_2 during 1h resulted in increased *ZAT12* transcript levels as compared to wild type plants. Gene expression of *CAT1*, not *CAT2* nor *CAT3*, is regulated by MKK1-mediated H_2O_2 production during different types of abiotic stress, such as drought and salt stress, and is related to ABA-signalling (Xing *et al*. 2007). In addition, transcript levels of *CAT1*, not *CAT2* nor *CAT3*, were reduced in 2-week-old *mkk1* knockout plants and increased in *MKK1* overexpressing plants as compared to wild type plants after 4h exposure to 300 mM NaCl, drought stress (dehydration of plants to 80% of their original fresh weight followed by incubation at 100% relative humidity at 25°C) or 0.1 mM ABA. Furthermore, production of H_2O_2 was abolished in *mkk1* knockout plants and higher in *MKK1* overexpressors treated with 300 mM NaCl, drought or 100 µM ABA. MKK1 mediates ABA-induced *CAT1* expression via MPK6 (Xing *et al*. 2008). As shown for *mkk1* knockout and *MKK1* overexpressing plants, *CAT1* transcript levels were reduced in 2-week-old *mpk6* knockout plants and elevated in *MPK6* overexpressors exposed to 100 µM ABA. ABA treatment also inhibited H_2O_2 accumulation in *mpk6* knockout plants.

1.5.4.3. MAPK cascades interfere with hormone signalling

Besides their role in physiological processes, ethylene and JA were originally identified as stress hormones essential for plant defence against a variety of abiotic and biotic stresses, such as ozone, UV radiation, mechanical stress, chemicals, metals, drought, extreme temperatures, insect and pathogen infection (Kendrick and Chang 2008, Wasternack and Kombrink 2010). In the past years, more and more evidence was presented to indicate that MAPK cascades can play a role in ethylene and JA signalling (Figure 1.5.).

1.5.4.3.1. Ethylene

MAPkinases have been reported to be involved in ethylene biosynthesis as well as in ethylene signalling. Several data indicate that MPK6 is involved in ethylene biosynthesis. It was reported in twelve-day-old *Arabidopsis* plants that MPK6, induced by flg22 or mechanical stress, is responsible for the phosphorylation of

two ACC synthases, ACS2 and ACS6. Conversion of S-adenosyl-L-methionine to ACC by ACC synthases is the rate-limiting and major regulatory step in stress-induced ethylene production. Phosphorylation of ACS2 and ACS6 stabilizes the ACS proteins, leading to an elevated ACS activity and consequently an elevated ACC and ethylene production (Liu and Zhang 2004). Also the MKK9-MPK3/6 pathway was shown to function in ethylene biosynthesis. Constitutive expression of MKK9-induced accumulation of ethylene through activation of MPK3/6 and consecutive positive regulation of *ACS2* and *ACS6* gene expression (Xu *et al*. 2008).

In contrast, other studies report that MAPKinases are involved in ethylene signal transduction rather than in ethylene biosynthesis. The function of the different components in the ethylene signalling pathway was recently reviewed by Shan *et al*. (2012) and will be discussed here briefly. Binding of ethylene to its receptor, ETR1 (Ethylene Response 1), releases CTR1 (Constitutive Triple Response 1), which in the absence of ethylene leads to degradation of EIN2 (Ethylene Insensitive 2), inhibiting downstream signal transduction. Therefore, binding of ethylene leads to accumulation of EIN2 activating a transcriptional cascade, initiated by the transcription factor EIN3 (Ethylene Insensitive 3), regulating transcription of ethylene-responsive genes (Shan et al. 2012). The first evidence for the involvement of a MAPK cascade in plant ethylene signalling came with the discovery of the first gene in the ethylene signal transduction pathway, *CTR1*, a negative regulator of ethylene responses. Indeed, the predicted protein sequence of CTR1 showed similarity to the mammalian Raf kinase, a MAPKKK (Kieber *et al*. 1993). More recently, Novikova *et al*. (2000) showed that a protein with similarities to a MAPK was activated by exogenous treatment of 6-week-old *Arabidopsis* plants with 1 μL L^{-1} ethylene during 1h. Treatment (10 min) of *Arabidopsis* wild type plants with 1mM ACC, the immediate precursor of ethylene in the biosynthesis pathway, identified the activation of MPK6. Mutant screenings showed that this activation of MPK6 by ACC is mediated by ETR1 and CTR1, but not by EIN2 or EIN3. These results place MPK6, as a positive regulator of ethylene responses, downstream of CTR1 and upstream of EIN2 in the ethylene signal transduction pathway (Ouaked *et al*. 2003). A study by Yoo *et al*. (2008) reported that the MAPK cascade MKK9-MPK3/6 functions downstream of CTR1, is activated upon binding of ethylene to

ETR1 and is able to phosphorylate and stabilize EIN3 leading to transcription of ethylene responsive genes. Yeast-2-hybrid and fluorescence resonance energy transfer identified the *in vitro* as well *in vivo* interaction of MPK6 with an ethylene response factor ERF104. The MPK6/ERF104 complex is disrupted by flg22-induced ethylene production, leading to phosphorylation and activation of ERF104. Microarray analysis of *ERF104* overexpressing plants identified the ERF104 stimulated targets as pathogenesis related (*PDF1.2*) or involved in further signal amplification of defence signalling (*MKK4*, *RBOHD*, *WRKY33*) (Bethke *et al*. 2009).

1.5.4.3.2. Jasmonate

Similar to ethylene, MAPKs are indicated to play a role in the biosynthesis of JA as well as in JA signalling. In case of salt stress, transcripts of two genes involved in JA biosynthesis, *OPR1* and *OPR2*, were shown to be more abundant in *wrky33* knockout plants, suggesting that MAPK cascades involving WRKY33 can downregulate JA biosynthesis during heat stress (Jiang and Deyholos 2009). Earlier, we mentioned that activation of transcription factors WRKY33 or ZAT12 by MAPK cascades induced gene expression of lipoxygenases, *LOX1* and *LOX4* respectively. Involvement of lipoxygenases in metal stress was also reported. Transcript levels of *LOX2* were significantly induced in leaves of *Arabidopsis* plants after short term exposure to 2 µM Cu or 5 µM Cd. In the roots, a metal-specific upregulation of *LOX3* and *LOX4* was observed after Cu exposure, whereas transcript levels of *LOX5* were specifically downregulated. In contrast, *LOX1* and *LOX6* gene expression was responsive to both Cd and Cu (Remans *et al*. 2010, Opdenakker *et al*. 2012: chapter 4). Furthermore, expression of *LOX1* and *LOX2* is compromised in roots and leaves of non-exposed *mpk6* knockout plants (Opdenakker *et al*., unpublished data). Lipoxygenases catalyze the first step in JA biosynthesis, namely the oxygenation of α-linolenic acid to hydroperoxides (Wasternack and Kombrink 2010). Earlier, LOX2 was identified as a lipoxygenase responsible for initiating JA biosynthesis upon wounding (Bell *et al*. 1995). Taken together, these data suggest that MAPK cascades can initiate JA biosynthesis via transcriptional control of LOXes.

On the other hand, studies reported that MAPKs would function in JA signal transduction. Leon *et al*. (2001) stated that reversible protein phosphorylation is

involved in JA signalling. JA-dependent induction of wound-inducible genes was stimulated by treatment of *Arabidopsis* plants with the protein kinase inhibitor staurosporin while treatment with the protein phosphatase inhibitor okadaic acid repressed this gene expression. These results suggest a negative regulation of the JA downstream pathway by protein kinase cascades. Petersen *et al*. (2000) showed that *mpk4* knockout plants are impaired in expression of *PDF1.2* and *THI1.2*, two JA-responsive genes. Moreover, treatment of 4-week-old *mpk4* knockout plants with 50 µM methyl-JA for 48h failed to induce *PDF1.2* or *THI1.2* transcript levels, indicating that MPK4 is involved in JA signalling. In addition, the MKK3-MPK6 pathway functions in JA signalling in *Arabidopsis* as a negative regulator of the downstream transcription factor MYC2. Treatment of *mkk3* and *mpk6* knockout as well as *MKK3* and *MPK6* overexpressing plants with 50 µM JA during 12h, showed that the MKK3/MPK6 pathway induced or reduced the transcript levels of *MYC2* respectively (Takahashi *et al*. 2007).

1.6. Conclusions

MAPK signalling plays a central role in plant metal stress responses. MAPkinases are activated by ROS production, induced upon metal stress, and convert the perception of metals to intracellular signals to the nucleus, where appropriate responses are initiated. However, MAPK cascades are not specific for a single stress condition. One MAPK cascade can be used by different biotic and abiotic stresses and interplay between different pathways is possible. In metal stress, the function of MAPK cascades is poorly understood while knowledge about metal signalling and more specific their downstream targets is essential for understanding plant responses to metal stress. Therefore, in future research it is important to focus on the functional analysis of MAPkinases in plant metal stress. For this purpose, mutants (knock-outs, overexpressors) of the different MAPKKKs, MAPKKs and MAPKs should be investigated under metal exposure in a single and multipollution context. Interaction between the different MAPKinase modules and the possible transcription factors activated by MAPKs can be identified by the use of functional protein microarrays (Popescu *et al*. 2009) or phosphoproteomics (van Bentem *et al*. 2008). Specific genes targeted by these transcription factors in their turn can be resolved using different molecular strategies. Better insight in plant metal stress responses and their regulation

opens future perspectives to investigate the complexity of signalling modules in plant responses facing a globally changing environment.

Chapter 2

Objectives

Cadmium (Cd) and copper (Cu) are naturally occurring in the environment. However, human activities elevated the concentration of these metals in the soil to toxic levels. Plants are able to accumulate these metals, spreading them into the food chain for animal and human consumption. Furthermore, this leads to the loss of food crops but also the loss of agricultural soils. Exposure of plants to toxic concentrations of Cd and Cu leads to well-studied physiological defects, such as growth retardation, disruption of photosynthetic processes and alterations in transpiration and respiration. Over the past years, more and more attention has been given to the cellular effects of metal exposure. Cadmium and Cu are known to produce reactive oxygen species (ROS) at the cellular level, which results in alterations in the cellular redox status in favour of the pro-oxidants, a condition also known as oxidative stress. Although ROS production in plant metal stress is described as harmful for the plant, ROS have also a positive role as signalling molecules in normal cell metabolism. Then, the question rises to what extent ROS production can be beneficial in the plant response to metal stress. Indeed ROS, and especially H_2O_2, are known to regulate the functioning of signalling pathways, like MAPKinases cascades. In normal plant processes, such as plant growth and development, as well as in different biotic and abiotic stress conditions, MAPK cascades are known to be activated by ROS to regulate the transcription of target genes involved in normal cell growth or in defence responses against these biotic and abiotic stresses. However, knowledge about the functioning of MAPK cascades in plant metal stress responses is rather scarce, but nevertheless essential for understanding plant responses to metal stress.

The main objective of this work was to study the specific function of MAPKinases, reported to be involved in other abiotic stresses, under metal stress. Specific emphasis was put on the question to what extent MAPKinases are regulating the oxidative stress response under Cd and Cu stress. And secondly, whether ROS signalling via MAPK cascades can lead to a better metal tolerance after long-term metal exposure.

To reach these objectives three main experimental topics were followed:

1. In the first part **(chapter 4)**, kinetic measurements were conducted to examine the early ROS production within the first day after Cd or Cu exposure. This was related with measurements of transcript levels of components from MAPK cascades and transcription factors, known to be regulated by ROS, to investigate ROS signalling after metal exposure.
2. The results of part 1 indicated a possible important role for the H_2O_2-inducible protein kinase OXI1 (oxidative-signal inducible 1) in metal-induced signalling. The function of OXI1 in Cu-induced oxidative stress was investigated in **chapter 5** using knockout mutants of *OXI1* in the Columbia background.
3. In the last part, the role of the MAPKinase MPK6, a downstream target of OXI1, was investigated in the metal-induced oxidative stress after Cu **(chapter 6)** and Cd **(chapter 7)** exposure. Special attention was given to transcription factors and pro- and antioxidative enzymes, which can be transcriptionally regulated via MPK6 after metal exposure.

Chapter 3

Materials and Methods

3.1. Plant material and treatment

Arabidopsis thaliana wild type, *oxi1* and *mpk6* mutant plants (ecotype Columbia-0) were used. Details about the mutants, such as the corresponding T-DNA insertion line and primers used for genotyping are given in Table 3.1.

The *Arabidopsis* plants were grown on hydroponics as described previously (Smeets *et al*. 2008b) except that purified sand was used as a substrate instead of rockwool. After three weeks, the plants were exposed to 2 µM $CuSO_4$ or 5 µM $CdSO_4$ during 2, 4, 6 or 24 hours. At harvest, leaves (entire rosette) and roots were removed, weighed and snap frozen in liquid nitrogen before storage at -80°C.

Table 3.1.: *Details about the used T-DNA knockout mutants.*

	oxi1	***mpk6***
Name	Gabi_355H08	SALK_073907
Mutation	T-DNA insertion line	T-DNA insertion line
Background	Columbia	Columbia
Genotype	homozygous	homozygous
Genotyping		
Forward primer	CTACAAATCTAGCTCCAAGAACGC	CTCTGGCTCATCGCTTATGTC
Reverse primer	GACCCTTGATTTCCTCAACGTTG	ATCTATGTTGGCGTTTGCAAC
T-DNA primer	GABI T-DNA border primer	LBb1.3
	CCCATTTGGACGTGAATGTAGACAC	ATTTTGCCGATTTCGGAAC
	→ to be used with the **forward** primer for detection of the mutant allele	→ to be used with the **reverse** primer for detection of the mutant allele

For root growth analysis, plants were grown on agar medium in vertically staked 12x12 cm Petri dishes (Dubrovsky and Forde 2012, Remans *et al*. 2012). For the root growth analysis of *oxi1* (chapter 5), the growth medium used was according to Remans *et al*. (2006) and consisted of 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM KH_2PO_4, 10 mM KNO_3, 0.5mM $(NH_4)_2SO_4$, 2.5 mM MES (2-[morpholino]ethane-sulphonic acid) (pH 5.7-5.8 adjusted with KOH), 50 mM NaFeEDTA, 50 µM

H_3BO_3, 12 µM $MnCl_2$, 1 µM $CuCl_2$, 1 µM $ZnCl_2$, and 0.03 µM NH_4MoO_4. The growth medium used for root growth analysis of *mpk6* (Chapter 6) was based on a 50-fold dilution of Gamborg's B5 medium (Zhang and Forde 1998) and contained 0.5 mM KNO_3, 0.02 mM $MgSO_4$, 0.02 mM $CaCl_2$, 0.022 mM NaH_2PO_4, 0.94 µM $MnSO_4$, 0.02 mM $(NH_4)_2SO_4$, 2.5 mM MES (pH 5.7-5.8), 90 nM KI, 0.97 nM H_3BO_3, 0.14 nM $ZnSO_4$, 2 nM $CuSO_4$, 20.6 nM Na_2MoO_4, 2.06 nM $CoSO_4$ and 3.6 µM $FeCl_3$. For germination, sucrose (5 g L^{-1}) was added to the medium. Medium for treatment plates was supplemented with $CuSO_4$ to become the following concentration range for the *oxi1* mutants: 0-20-25-30-35-40-45-50-60 µM Cu and for the *mpk6* mutants: 0-1-2-5-10-15-20 µM Cu. In the root growth experiment using *oxi1* knockouts, higher amounts of $CuSO_4$ were applied to the growth medium because the concentrations of the essential nutrients used in this medium were higher. *Arabidopsis* seeds were surface sterilized in a cone-shape folded filterpaper (Whatmann nr. 542, closed with a paper clip) for 1 min in a 0.1% sodium hypochlorite solution (diluted in dH_2O from Sigma Aldrich Sodium hypochlorite solution 10-13%) containing Tween 80 (1 drop per 100 mL), followed by 4 washes (15s) with a small amount of sterile water and 4 washes of 5 min with a larger volume of sterile water and finally drying in a laminar air flow. Sterilized seeds were planted with a sterile toothpick in 12 cm x 12 cm transparent Petri dish on 40 ml of solid (1% agar) germination medium. After storing for 2 d at 4°C in the dark, plates were incubated vertically in a growth chamber at 22°C with a 12h/12h light/dark regime and a light intensity of 165 µmol m^{-2} s^{-2}. After one week, a homogenous set of plants growing on the surface of the agar were transferred to treatment plates containing different concentrations of Cu (5 plants per plate) and were incubated for another week. Primary root tip position was marked every 24h on the outside of the plates, and plates were scanned after 1 week using a conventional flatbed scanner (CanonScan 4400F). Root growth was analyzed using the Optimas image analysis software (MediaCybernetics, Silver Spring, MD).

3.2. Element analysis

Leaf and root samples were taken for the determination of an elemental profile. Roots were washed for 10 min in ice-cold 1mM $Pb(NO_3)_2$ and twice with ice-cold distilled H_2O to exchange surface-bound Cu; leaves were rinsed with distilled

water. Subsequently, plant samples were dried at 80°C, weighed, and digested in HNO_3 (70-71%) using a heat block. The concentrations of Cu, Zn, K, Ca, Fe, Mg, Mn, S and P were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES). Blanks (only HNO_3) and a standard sample [NIST Spinach (1570a)] were analyzed for reference purposes.

3.3. Hydrogen peroxide measurement

Approximately 150 mg frozen (-80°C) leaf or root sample (in 2 ml microcentrifuge tubes) was ground thoroughly using two stainless steel beads (2 mm diameter) in each sample and the Retsch Mixer Mill MM2000. The ground tissue was homogenized in 750 µl 0.2 M $HClO_4$ and incubated on ice for 5 min. After centrifugation (10 min, 10 000*g*, 4°C), a pH between 8 and 8.5 was obtained in 410 µl supernatant by adding 490 µl 0.2 M NH_4OH. The pH adjusted supernatant was centrifuged during 2 min (3 000*g*) and incubated on ice. The hydrogen peroxide (H_2O_2) in the supernatant was bound to and eluted from poly-prep chromatography columns filled with AG1-X8 Resin (Bio-Rad Laboratories).

The concentration of H_2O_2 in leaves and roots was then measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carisbad/California, USA). This is a sensitive, one-step fluorometric assay that uses 10-acetyl-3,7-dihydroxyphenoxazine to detect H_2O_2 in biological samples. Fifty µl of the eluate was measured together with a H_2O_2 standard curve (also bound to and eluted from the poly-prep prefilled chromatography columns) ranging from 0 to 80 µM (as reference for leave samples) or 0 to 50 µM (as reference for root samples). Fluorescence of the red-fluorescent oxidation product, resorufin, was measured at 590 nm after excitation at 530 nm using the FLUOstar OMEGA (BMG LABTECH, Isogen Life Sciences, De Meern, The Netherlands). The level of H_2O_2 was calculated according to the standard curve and corrected for the fresh weight and the dilution made during the procedure.

3.4. Determination of lipid peroxidation

The extent of lipid peroxidation in leaves and roots was estimated by measuring the concentration of thiobarbituric acid (TBA)-reactive compounds spectro-

photometrically. Samples (100 mg) were homogenized in 2 ml 0.1% trichloroacetic acid (TCA) buffer and after centrifugation, 2 ml 0.5% TBA buffer was added to the supernatant. Subsequently, the samples were incubated at 95°C during 30 min and rapidly cooled down on ice. After centrifugation (10 min, 20 000*g*), the absorbance of the supernatant was measured at 532 nm and at 600 nm for the aspecific absorbance. The content of TBArm in leaves and roots was calculated according to the law of Lambert-Beer (ε = 155 mM^{-1} cm^{-1}) and corrected for the aspecific absorbance, the fresh weight used and the dilutions made during the produce.

3.5. Determination of glutathione content

Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were analyzed according to the spectrofotometric microplate reader method described by Queval and Noctor (2007). Frozen leaf or root tissue (150 mg) was ground thoroughly in liquid nitrogen, homogenized in 500 µl of 0.2 M HCl and centrifuged during 15 min (16 000*g*, 4°C). Then a pH between 4 and 5 was obtained by adding 30 µl of 0.2 M NaH_2PO_4 (pH 5.6) and ca. 200 µl of 0.2 M NaOH to 300 µl supernatant. Measurement of GSH and GSSG is based on the glutathione reductase (GR)-dependent reduction of 5,5-dithiobis(2-nitro-benzoic acid) (DTNB), monitored at 412 nm. When using the pH adjusted supernatant from above, the method measures the total amount of glutathione in the sample that is GSH plus GSSG. For measuring GSSG, 130 µl of the pH adjusted supernatant was incubated with 1.3 µl 2-vinyl-pyridine (2-VP), which precipitates all GSH present in the sample, during 30 min at room temperature. After centrifugation (15 min, 16 000*g*, 4°C) to precipitate 2-VP, the supernatant was transferred to clean tubes and centrifuged again. To measure total glutathione, 10 µl of the supernatant was added in triplicate to a 96-well plate containing 100 µl of 0.2 M NaH_2PO_4–10 mM EDTA buffer (pH 7.5), 10 µl of 10 mM NADPH, 10 µl of 12 mM DTNB and 60 µl of dH_2O. The reaction was started by the addition of 10 µl GR and after 20 s of automatic mixing by shaking, the rate of DTNB reduction was monitored during 5 min. Sample concentrations of total glutathione were calculated relative to a standard curve ranging from 0 to 1 nmol GSH, measured in duplicate in the same plate, and were corrected for GSH-independent reduction of DTNB by subtraction of the mean value of

duplicate blank assays (0 nmol GSH). GSSG was measured by the same principle after incubation with 2-VP to complex GSH. 20 µl of the final supernatant was measured in triplicate as described above, together with GSSG standards also subjected to incubation with 2-VP and ranging from 0 to 200 pmol. Concentrations of GSSG were calculated as for total glutathione. Finally, the standard curve corrected GSH and GSSG concentrations were corrected for the fresh weight and amounts of 0.2 M NaOH used, as well as for the dilutions made during the procedure.

3.6. Determination of enzyme activities

Frozen leaf (200 mg) or root (100 mg) tissue was homogenized in 2 ml ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1mM EDTA, 1 mM dithiotreitol and 4% insoluble polyvinylpyrrolidone. The homogenate was squeezed through a nylon mesh and after centrifugation (10 min, 20 000*g*, 4°C), enzyme activities were measured spectrophotometrically in the supernatant at 25°C.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured at 550 nm by following the inhibition of cytochrome C mediated neutralization of $O_2^{\bullet-}$. The $O_2^{\bullet-}$ generating system consists of xanthine oxidase and xanthine. In the blanc cuvet, cytochrome c will be reduced by the formed superoxide radicals. Addition of the plant extract results in a disproportionation of the superoxide radicals and hence an inhibition of the reduction of cytochrome c, which is a measure to define SOD capacity. The amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% is defined as 1 unit of activity. For this reaction, 100 µl of leaf (1/20 diluted) or root (1/5 diluted) extract was added to 590 µl 50 mM KH_2PO_4 buffer (pH 7.8), 100 µl 1mM EDTA, 0.5 mM xanthine and 100 µl 0.1 mM cytochrome C. The reaction was started by the addition of 10 µl xanthine oxidase (XOD).

H_2O_2 removal by cell wall bound and other peroxidases (POD) was analysed by measuring the oxidation of the chemical substrates syringaldazine and guaiacol. The activity of SPOD (EC 1.11.1.7) was determined at 530 nm in 33 µl of supernatant incubated with 850 µl 0.1 M Tris buffer (pH 7.5), 100 µl 10 mM H_2O_2 and 17 µl syringaldazine. GPOD (EC 1.11.1.7) activity was measured at 436 nm by the addition of 50 µl leaf or root (1/5 diluted) supernatant and 100 µl 18 mM guaiacol to 750 µl 0.1 M KH_2PO_4 buffer (pH 7.0) and 100 µl 6 mM H_2O_2.

Removal of H_2O_2 can also occur by catalase (CAT, EC 1.11.1.6) and was determined at 240 nm by adding 50 µl leaf or 100 µl root extract to respectively 780 or 730 µl 0.1 M KH_2PO_4 buffer (pH 7.0) and 170 µl 5 mM H_2O_2.

The activity of glutathione reductase (GR, EC 1.6.4.2) was determined by the reduction of GSSG at 340 nm. Therefore, 150 µl leaf or root extract was added to 815 µl 0.1 M Tris- 1 mM EDTA buffer (pH 8.0), 17.5 µl GSSG and 17.5 µl NADPH.

Activities of all enzymes, except for SOD, were calculated according to the law of Lambert-Beer (ε_{SPOD} = 11.6 $M^{-1}cm^{-1}$, ε_{GPOD} = 25.5 $M^{-1}cm^{-1}$, ε_{GR} = 6.22 $M^{-1}cm^{-1}$, ε_{CAT} = 40 $M^{-1}cm^{-1}$) and corrected for the fresh weight used and the dilutions made during the procedure.

3.7. Gene expression analysis

Frozen (-80°C) plant tissue (less than 100 mg) in 2 ml microcentrifuge tubes was ground thoroughly under frozen conditions using two stainless steel beads (2 mm diameter) in each sample and the Retsch Mixer Mill MM2000. RNA was extracted from the disrupted tissue using the miRVANA Total RNA Isolation or the RNAqueous Small Scale Phenol-Free Total RNA Isolation procedure (Ambion). The RNA concentration and purity were measured spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies, Isogen Life Sciences). The integrity of the RNA was checked with the Agilent-2100 BioAnalyzer and RNA 6000 NanoChips (Agilent Technologies). Contaminating gDNA was removed by incubating 1 µg of the RNA sample in gDNA wipeout buffer at 42 °C for 2 min (QuantiTect Reverse Transcription Kit, Qiagen) or in TURBO DNase at 37 °C for 25 min (TURBO DNA-free kit, Ambion). First strand cDNA synthesis was performed using a combination of oligo(dT)-primers and random hexamers according to the manufacturer's instructions using the QuantiTect Reverse Transcription Kit (Qiagen) or the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Subsequently, the cDNA sample was diluted 10-fold in 1/10 TE-buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Quantitative PCR was performed with the 7500 Fast real-time PCR cycler (Applied Biosystems) and SYBR green chemistry. PCR reactions were carried out in a total volume of 10 µl, containing 2 µl cDNA sample, 5 µl Fast SYBR green Master Mix (Applied Biosystems) and 300 nM of each primer. Primer sequences

for reference genes were according to Remans *et al*. (2008). Other primers were designed using Primer Express v2.0 (Applied Biosystems). Specificity of the primers was checked *in silico* using TAIR BLAST (www.arabidopsis.org) and after qPCR by verifying the occurrence of single peaks on the melting curve. The amplification efficiencies of all primer sets were investigated by a 2-fold serial dilution over 6 dilution points and were approved when they were greater than 80%. Genes and primer sequences for RT-qPCR are listed in Table 3.2.

Table 3.2.: *Primer sequences of reference genes and genes of interest.*

Locus	Gene	Forward primer	Reverse primer
Reference genes			
At2g28390	*SAND family protein*	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
At4g26410	*expressed*	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
At4g34270	*TIP41-like*	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
At5g08290	*mitosis protein YSL8*	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT
At5g15710	*F-box protein*	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA
At5g25760	*UBC*	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
At5g55840	*PPR gene*	AAGACAGTGAAGGTGCAACCTTACT	AGTTTTTGAGTTGTATTTGTCAGAGAAAG
At5g60390	*Ef1α*	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA
Other genes			
At1g55020	*LOX1*	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC
At3g45140	*LOX2*	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC
At5g51060	*RBOHC*	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC
At5g47910	*RBOHD*	AACTCTCCGCTGATTCCAACG	TGGTCAGCGAAGTCTTTAGATTCCT
At1g08830	*CSD1*	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
	hnCSD1	ACTGTTGGAGATGATGGTATGCCT	GAGAGAGTAGCGAAATTTGATGCAA
At2g28190	*CSD2*	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
	hnCSD2	TCACTATGACTTAGGCTGCGATTG	GATGGTCCGAATTTGCGATTAA
At4g25100	*FSD1*	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
At3g10920	*MSD1*	ATGTTTGGGAGCACGCCTAC	AACCTCGCTTGCATATTTCCA
At1g20630	*CAT1*	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
At4g35090	*CAT2*	AACTCCTCCATGACCGTTGGA	TCCGTTCCCTGTCGAAATTG
At1g20620	*CAT3*	TCTCCAACAACATCTCTTCCCTCA	GTGAAATTAGCAACCTTCTCGATCA
At1g07890	*APX1*	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
At1g09000	*ANP1*	AAGAGAGGACACTGCTCGTGG	TTGCGTCTGTTGCTCTTGAAG
At3g25250	*OXI1*	CGATTATTGTCCGGGACAGA	CTAATACAAGCTCCGCCGC
At3g45640	*MPK3*	GACGTTTGACCCCAACAGAA	TGGCTTTTGACAGATTGGCTC
At2g43790	*MPK6*	TAAGTTCCCGACAGTGCATCC	GATGGGCCAATGCGTCTAA
At4g01250	*WRKY22*	AAACCCATCAAAGGTTCACCA	GGGTCGGATCTATTTCGCTC
At2g30250	*WRKY25*	GAAAGATCCGCAGCAGACG	TCCCAATAATTTCACGAGCG
At4g23550	*WRKY29*	CATGGGCGTGGCGTAAATA	TTGTTTTCTTGCCAAACACCC
At5g59820	*ZAT12*	GTGCGAGTCACAAGAAGCCTAACA	GCGACGACGTTTTCACCTTCTTCA
At5g44420	*PDF1.2*	TTTGCTGCTTTCGACGCAC	GCATGCATTACTGTTTCCGCA
At2g03445	*pri-MIR398a*	AGAAGAAGAGAAGAACAACAGGAGGTG	ATTAGTAAGGTGAAAAAATGG
At5g14545	*pri-MIR398b*	AGTAATCAACGGCTGTAATGACGCTAC	TGACCTGAGAACACATGAAAACGAGAG
At5g14565	*pri-MIR398c*	TCGAAACTCAAACTGTAACAGTCC	ATTTGGTAAATGAATAGAAGCCACGGGCCACG
At5g44070	*PCS1*	TGGTGTTGAATGCTCTTTCTATCG	GGTTCGCAGCAATCCAACAT
At1g07600	*MT1a*	AACTGTGGATGTGGCTCCTC	CAGTTACAGTTTGACCCACAGC
At1g07610	*MT1c*	GCATGGTCTCAAACCAAGGA	TACGCAACACAATGCCAAGT
At3g09390	*MT2a*	ACCCTGACTTGGGATTCTCC	GCGTTGTTACTCTCCCCTGA
At5g02380	*MT2b*	ACTCTTGTCCTCGGTGTTGC	TTGCACTTGCAGTCAGATCC
At3g15353	*MT3*	TCGACATCGTCGAGACTCAG	CACTTGCAATTTGCGTTGTT

Relative quantities were calculated as $2^{-\Delta Cq}$ and normalized to a normalisation factor based on the geometric mean of the expression level of multiple reference genes. The choice of reference genes used, was based on GeNorm (v3.4) and Normfinder (v0.953) reevaluation of the reference genes selected by Remans *et al*. (2008) for Cd and Cu exposure after 24h. 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.

3.8. Clustering analysis of the gene expression data

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

3.9. Statistical analysis

The datasets were analyzed using the two-way ANOVA GLM procedure in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). When time-course measurements were performed, the two-way ANOVA was used to calculate statistical significance within each time point, since we did not compare the different time points with each other. The Tukey adjustment for multiple comparisons was applied to obtain corrected P-values. Normal distribution of the data was checked using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Transformation (log(x), $\sqrt{x}$, e^x, 1/x) of the data was applied when necessary to approximate normality. Homoscedasticity of the data was evaluated by a residue plot.

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 leaves	Reference genes roots
Cu/Cd kinetics	4	miRVANA Total RNA Isolation kit	QuantiTect Reverse Transcription kit	**Cu**	**Cu**
				At4g26410	*At2g28390*
				At4g34270	*At4g34270*
				At5g25760	*At4g26410*
				At2g28390	*At5g25760*
				At5g55840	*At5g08290*
				At5g08290	
				At5g15710	
				Cd	**Cd**
				At2g28390	*At2g28390*
				At5g15710	*At5g15710*
				At5g08290	*At5g08290*
oxi1/Cu	5	miRVANA Total RNA Isolation kit	QuantiTect Reverse Transcription kit	*At5g08290*	*At5g08290*
				At5g15710	*At5g15710*
					At5g25760
					At4g34270
					At4g26410
mpk6/Cu	6	RNAqueous Small Scale Phenol-Free Total RNA Isolation kit	TURBO DNA-free kit High-Capacity cDNA Reverse Transcription Kit	**Hydroponics**	**Hydroponics**
				At2g28390	*At2g28390*
				At5g15710	*At5g15710*
				At5g60390	*At5g60390*
				VAPs	
				At2g28390	
				At5g15710	
				At3g10920	
mpk6/Cd	7	RNAqueous Small Scale Phenol-Free Total RNA Isolation kit	TURBO DNA-free kit High-Capacity cDNA Reverse Transcription Kit	*At5g15710*	*At5g15710*
				At5g08290	*At5g08290*
				At2g28390	*At2g28390*

Chapter 4

Exposure of *Arabidopsis thaliana* to Cd or Cu excess leads to oxidative stress mediated alterations in MAPKinase transcript levels

Kelly Opdenakker, Tony Remans, Els Keunen, Jaco Vangronsveld, Ann Cuypers (2012). Exposure of *Arabidopsis thaliana* to Cd or Cd excess leads to oxidative stress mediated alterations in MAPKinase transcript levels. Environmental and Experimental Botany, 83, 53-61.

Abstract

Metals, like cadmium (Cd) and copper (Cu), have the ability to induce the production of reactive oxygen species (ROS) at the cellular level. It is widely known that these ROS can cause irreversible damage to cellular components, like DNA, proteins and lipids. On the other hand, ROS can also act as signalling molecules and in this way they play an essential role in many normal physiological processes, but also in defence responses against stress. Reactive oxygen species signalling in plants uses mitogen-activated protein kinase (MAPK) pathways leading to the transcriptional control of target genes involved in the scavenging or production of ROS. Here, oxidative signalling induced by exposure to excess Cd or Cu was investigated in relation to anti-oxidative defence responses to these metals. Three-week old *Arabidopsis thaliana* plants were exposed to environmentally realistic concentrations of Cu and Cd and immediate responses were measured at the level of hydrogen peroxide (H_2O_2) content, lipid peroxidation and transcript levels of genes involved in ROS homeostasis and signalling. Our findings show immediate (after 2h exposure) effects in the roots following Cu exposure, whereas effects in the leaves were generally more delayed. Effects of Cd exposure in leaves and roots were observed only after 24h exposure. On one hand, exposure of roots to Cu leads via activation of NADPH oxidases and Fenton reactions to H_2O_2 production that can induce MAPK and consequently oxylipin signalling to control the cellular redox status. On the other hand, conversion of H_2O_2 to the more damaging hydroxyl radical by Fenton and Haber-Weiss reactions can initiate lipid peroxidation leading to membrane damage. In roots exposed to elevated Cd concentrations only oxidative signalling was initiated, possibly via NADPH oxidase - mediated ROS production. In leaves, time-dependent activation of MAPK and oxylipin signalling was seen after exposure to both metals, Cu or Cd, independent of changes in H_2O_2 content.

4.1. Introduction

Toxic metals, like cadmium (Cd) and copper (Cu), are known to induce stress effects in plants (DalCorso *et al*. 2008, Yruela 2009, Cuypers *et al*. 2011a). Cadmium is a non-essential element for plants and therefore toxic, even at low concentrations. In contrast, Cu is an essential trace element for the plant but can also become toxic for plants when locally present at increased concentrations, for example in polluted environments.

At the cellular level, both Cd and Cu may induce oxidative stress (Cuypers *et al*. 2009, Smeets *et al*. 2009, Cuypers *et al*. 2011a). Oxidative stress is a disturbance of the cellular redox status in favour of the pro-oxidants and is often occurring in stress conditions (Mittler 2002). Copper is a redox-active metal that can produce reactive oxygen species (ROS) directly via Fenton and Haber-Weiss reactions (Kehrer 2000). Cadmium on the other hand, is not redox-active and causes oxidative stress via indirect mechanisms, like interactions with enzymes of the antioxidative defence system (Das *et al*. 1997, Cuypers *et al*. 2009). Even though excessive ROS production is linked to stress conditions, controlled levels of ROS are also essential for normal cell metabolism, which requires fine-tuning of subcellular ROS levels by the antioxidative defence system of the cell, consisting of enzymes and metabolites (Mittler *et al*. 2004, Halliwell 2006). For example, hydrogen peroxide (H_2O_2) acts as a signalling molecule in mediating defence responses against biotic and abiotic stresses (Torres and Dangl 2005) but has also a role during normal cell growth and development (Foreman *et al*. 2003). As a signalling molecule, H_2O_2 can modulate the activities of many components in cell signalling, such as protein phosphatases, protein kinases and transcription factors (Miller *et al*. 2008, Quan *et al*. 2008). H_2O_2 is involved in the activation of the mitogen-activated protein kinase kinase kinase (MAPKKK) *Arabidopsis NPK1-like protein kinase 1* (ANP1) and the serine/threonine kinase *Oxidative signal-inducible 1* (OXI1) (Suzuki *et al*. 1999, Kovtun *et al*. 2000, Rentel *et al*. 2004). Both kinases induce a phosphorylation cascade involving two stress-responsive mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 (Kovtun *et al*. 2000, Rentel *et al*. 2004). MAPK cascades have the possibility to regulate gene transcription by activation or repression of transcription factors (Colcombet and Hirt 2008). From MPK3 and MPK6, it is known that activation of these MAPKs in *Arabidopsis* leads to induction of the transcription factors

WRKY22 and WRKY29 (Asai *et al*. 2002). WRKY proteins bind to W-box DNA elements (containing a TGAC core sequence) found in the promoters of many defence-related genes (Pandey and Somssich 2009). WRKY25 is another transcription factor of the WRKY family and studies point out a role for WRKY25 in the modulation of gene transcription during heat stress or in plant defence against pathogenic bacteria like *Pseudomonas syringae* (Zheng *et al*. 2007, Li *et al*. 2009). The zinc-finger protein ZAT12 is a transcription factor in *Arabidopsis* that in response to H_2O_2 results in the enhanced expression of oxidative stress- and light stress-responsive transcripts, one of them being *Ascorbate peroxidase 1* (APX1). Therefore it can be suggested that ZAT12 plays a central role in reactive oxygen and abiotic stress signalling in *Arabidopsis* (Davletova *et al*. 2005a,b).

Several authors reported the involvement of MAPK signalling in case of metal stress. In *Medicago sativa* roots as well as in rice roots, the orthologues of *Arabidopsis* MPK3 and MPK6, SAMK/SIMK and OsMPK3/MPK6 respectively, were activated after exposure to excess Cd or Cu ions (Jonak *et al*. 2004, Yeh *et al*. 2007). They proposed that Cd and Cu both induce MAPKs via ROS generation, but make use of distinct signalling pathways depending on the type of ROS generated. In addition, Wang *et al*. (2010) demonstrated in *Zea mays* that *ZmMPK3* transcript levels are induced after exposure to high concentrations (500 µM) $CdCl_2$. In *Arabidopsis*, it was proven that MPK3 and MPK6 are activated in response to short-term exposure (less than 1h) to $CdCl_2$ concentrations as low as 1 µM, via the accumulation of ROS (Liu *et al*. 2010). However, little is known about the exact signalling pathways and the downstream targets of these pathways operating in plants under metal stress.

Since Cu and Cd are known to induce ROS production and the above-mentioned MAPK cascades can be activated by H_2O_2, we hypothesize that H_2O_2 and MAPK cascades are also induced by plant exposure to Cu and Cd and that they are involved in the subsequent stress. Therefore, the aim of this study was (1) to examine the responses of known MAPK cascades under metal stress and (2) to relate their behaviour to the redox status of the cell. The effects of a short-term exposure (24h) to sublethal Cd and Cu concentrations on the redox status of the cell in *Arabidopsis thaliana* were described earlier (Smeets *et al*. 2009; Cuypers

et al. 2011a). Therefore a kinetic experimental set-up was conducted immediately after the onset of the exposure.

4.2. Results

4.2.1. Exposure of Arabidopsis to excess Cu affects root weight

Arabidopsis thaliana seedlings were grown on hydroponics for three weeks and subsequently exposed to 2 µM $CuSO_4$ or 5 µM $CdSO_4$ during 2, 4, 6 or 24h. Plants exposed for 24h to Cu showed a significantly lower root fresh weight than control plants, as well as a decreased rosette weight, although not statistically significant (Figure 4.1.). When the roots were dried for a couple of days at 80°C and the dry weight was analyzed, root weight of control and Cu-exposed plants showed no differences (data not shown). In contrast, exposure of *A. thaliana* to Cd did not cause any changes in leaf or root weight.

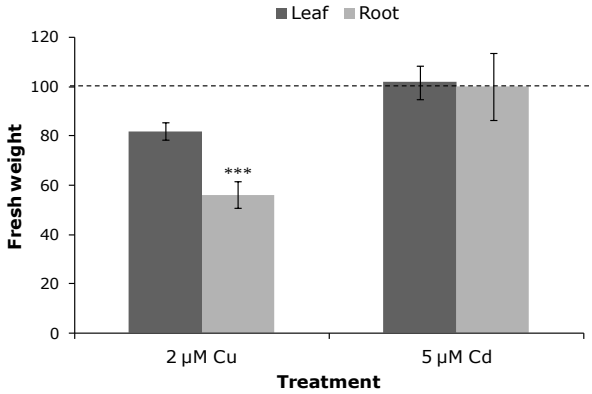


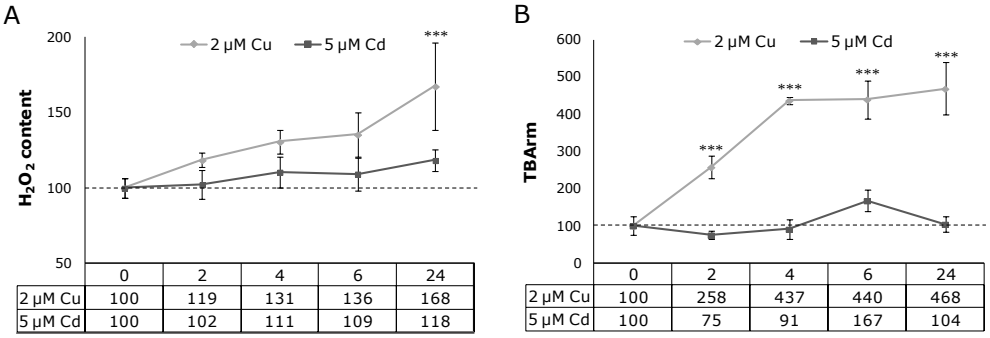
Figure 4.1.: *Leaf and root weight, relative to the non-exposed plants (= 100%), of 3-week old Arabidopsis thaliana plants exposed to 2 µM $CuSO_4$ or 5 µM $CdSO_4$ during 24h. Values are mean ± S.E. of at least 20 biologically independent replicates (significance level: ***: $p < 0.001$).*

4.2.2. Exposure of Arabidopsis to excess Cu increases H_2O_2 levels and induces lipid peroxidation

No significant differences in H_2O_2 content were found between leaves of control and Cu- or Cd-exposed plants, although an increasing trend in H_2O_2 content was observed in the leaves after 24h exposure to Cu or Cd (data not shown). However, in roots of Cu-exposed plants, H_2O_2 levels seem to be enhanced after already 2h of exposure (Figure 4.2.A). In Cd-exposed roots, no significant differences in H_2O_2 levels were seen, although an increasing trend could be

observed after 24h exposure to Cd (Figure 4.2.A). When the H_2O_2 contents over all time points were considered in global, the H_2O_2 contents of Cu-exposed plants were significantly higher than the H_2O_2 contents of non-exposed ($p < 0.0001$) or Cd-exposed roots ($p < 0.0017$) (Figure 4.2.A).

Lipid peroxidation was determined in leaves and roots of Cu- and Cd-exposed plants by determining thiobarbituric acid reactive metabolites (TBArm). In leaves of Cu- and Cd-exposed plants, no differences in lipid peroxidation were detected (data not shown). In contrast, a significant increase in TBArm was observed in roots after already 2h exposure to Cu (Figure 4.2.B). This lipid peroxidation reached its maximum after 4h and then stabilized at this level for the remaining experimental period. In roots of Cd-exposed *Arabidopsis* seedlings, a transient but not statistically significant increase in lipid peroxidation was observed at 6h exposure. For the remaining of the experimental period, TBArm levels were equal at the control level (Figure 4.2.B).



	0	2	4	6	24
2 µM Cu	100	119	131	136	168
5 µM Cd	100	102	111	109	118

	0	2	4	6	24
2 µM Cu	100	258	437	440	468
5 µM Cd	100	75	91	167	104

Figure 4.2.: *H_2O_2 concentration (A) and lipid peroxidation (B), relative to the non-exposed plants (= 100%), in roots of Arabidopsis thaliana plants exposed to 2 µM $CuSO_4$ or 5 µM $CdSO_4$ during 0, 2, 4, 6 and 24h. Values are mean ± S.E. of at least 5 biologically independent replicates (significance level: ***: $p < 0.001$).*

4.2.3. Expression of oxidative stress and signalling-related genes in Arabidopsis seedlings exposed to excess Cu or Cd

Transcript levels of ROS-producing enzymes (lipoxygenases, NADPH oxidases), antioxidative defence enzymes (superoxide dismutases, ascorbate peroxidase, catalases) and signalling components (MAPkinases, transcription factors) were determined in roots and leaves of *Arabidopsis* seedlings under control conditions (Table 4.1.) or exposed to Cu or Cd (Table 4.2. and 4.3.). In general, analysis of

gene expression of non-exposed plants in time revealed significant circadian changes in gene expression, especially in the leaves. Significant responses were already observed from 2h in roots of Cu-exposed plants, whereas in leaves of Cu-exposed plants, delayed responses were observed. In roots and leaves of Cd-exposed plants significant responses were observed only after 24h.

4.2.3.1. Exposure of Arabidopsis to excess Cu or Cd changes gene expression of ROS-producing enzymes in a time-dependent manner

In roots of Cu-exposed plants (Table 4.2.), gene expression of the lipoxygenase 1 (LOX1) was significantly increased after 4h, increased 30-fold after 6h and remained elevated after 24h. Also exposure to Cd resulted in an almost 10-fold increase of *LOX1* expression, but in contrast to the fast response to Cu, in case of Cd a response was observed only after 24h. The NADPH oxidases *RBOHC* and *RBOHD* in roots showed significantly increased gene expressions at 2, 4 and 6h after starting exposure to Cu. However, after 24h exposure to Cu, the *RBOHC* expression was reduced and was even significantly lower as compared to control plants. In contrast, after 24h, gene expression of *RBOHD* was still 10 times higher in Cu-exposed plants. In roots of Cd-exposed plants, a significant induction of gene expression was only observed after 24h exposure for *RBOHD.*

In leaves of Cu-exposed plants, expression of the lipoxygenase 2 (LOX2) gene was initially significantly reduced after exposure but a significant increase was observed from 6h on (Table 4.3.). An increase in *LOX2* expression was also observed in Cd-exposed plants but this was only seen after 24h. With regard to the NADPH oxidases, only *RBOHD* expression in leaves was significantly increased after 6 and 24h exposure to Cu, whereas both *RBOHC* and *RBOHD* were significantly upregulated after 24h exposure to Cd.

Table 4.1.: *Transcript levels of ROS-producing, antioxidative and signalling enzymes, and transcription factors relative to the non-exposed plants at time point 0 (= 1), in roots and leaves of 3-week-old non-exposed A. thaliana plants. Values are mean ± S.E. of at least 5 biologically independent replicates (significance levels: downregulation: p < 0.05 | p<0.01 | p < 0.001 ; upregulation: p < 0.05 | p<0.01 | p < 0.001).*

	ROOTS					LEAVES				
Gene	0h	2h	4h	6h	24h	0h	2h	4h	6h	24h
LOX1/2	1.00 ± 0.06	1.09 ± 0.06	1.14 ± 0.16	1.19 ± 0.16	1.10 ± 0.19	1.00 ± 0.07	3.59 ± 0.58	6.76 ± 1.76	4.41 ± 0.82	0.96 ± 0.08
RBOHC	1.00 ± 0.03	0.76 ± 0.03	0.57 ± 0.06	0.74 ± 0.10	0.98 ± 0.09	1.00 ± 0.21	0.99 ± 0.29	0.49 ± 0.11	0.88 ± 0.31	0.89 ± 0.09
RBOHD	1.00 ± 0.07	0.84 ± 0.09	0.86 ± 0.10	0.92 ± 0.19	1.22 ± 0.17	1.00 ± 0.02	0.71 ± 0.05	0.62 ± 0.04	0.51 ± 0.05	0.78 ± 0.06
CSD1	1.00 ± 0.03	0.98 ± 0.03	0.94 ± 0.03	0.99 ± 0.05	0.88 ± 0.03	1.00 ± 0.07	1.30 ± 0.04	1.25 ± 0.10	1.22 ± 0.05	0.78 ± 0.05
CSD2	1.00 ± 0.04	0.86 ± 0.03	0.82 ± 0.02	0.86 ± 0.03	0.94 ± 0.06	1.00 ± 0.07	1.17 ± 0.06	0.99 ± 0.10	0.94 ± 0.09	1.10 ± 0.14
FSD1	1.00 ± 0.08	1.13 ± 0.13	1.49 ± 0.29	1.52 ± 0.30	2.56 ± 0.42	1.00 ± 0.08	0.50 ± 0.05	0.47 ± 0.06	0.37 ± 0.05	0.92 ± 0.09
APX1	1.00 ± 0.03	0.99 ± 0.03	0.80 ± 0.06	0.86 ± 0.07	0.95 ± 0.07	1.00 ± 0.03	1.00 ± 0.05	1.03 ± 0.06	0.84 ± 0.04	1.16 ± 0.09
CAT1	1.00 ± 0.05	1.24 ± 0.11	1.67 ± 0.07	2.37 ± 0.27	0.86 ± 0.08	1.00 ± 0.03	1.65 ± 0.14	4.15 ± 0.37	4.87 ± 0.35	0.92 ± 0.02
CAT2	1.00 ± 0.06	0.91 ± 0.05	0.73 ± 0.10	0.82 ± 0.12	0.75 ± 0.09	1.00 ± 0.03	0.36 ± 0.03	0.17 ± 0.01	0.08 ± 0.01	0.93 ± 0.04
CAT3	1.00 ± 0.05	1.23 ± 0.13	1.40 ± 0.16	1.92 ± 0.43	1.04 ± 0.21	1.00 ± 0.06	2.14 ± 0.13	5.38 ± 0.44	7.22 ± 0.36	0.89 ± 0.03
ANP1	1.00 ± 0.04	1.11 ± 0.05	0.84 ± 0.02	0.88 ± 0.07	0.81 ± 0.04	1.00 ± 0.03	1.30 ± 0.07	1.17 ± 0.07	1.37 ± 0.06	0.92 ± 0.08
OXI1	1.00 ± 0.07	0.74 ± 0.04	0.59 ± 0.06	0.53 ± 0.07	0.74 ± 0.05	1.00 ± 0.12	0.53 ± 0.06	0.40 ± 0.05	0.47 ± 0.07	0.83 ± 0.06
MPK3	1.00 ± 0.05	0.99 ± 0.11	1.15 ± 0.08	1.23 ± 0.11	1.07 ± 0.11	1.00 ± 0.10	0.94 ± 0.07	0.81 ± 0.08	0.85 ± 0.05	0.84 ± 0.04
MPK6	1.00 ± 0.02	0.89 ± 0.02	0.93 ± 0.05	0.94 ± 0.07	0.98 ± 0.05	1.00 ± 0.03	0.94 ± 0.04	0.89 ± 0.03	0.86 ± 0.02	0.76 ± 0.05
WRKY22	1.00 ± 0.06	0.93 ± 0.03	0.82 ± 0.06	0.77 ± 0.10	0.97 ± 0.12	1.00 ± 0.03	0.68 ± 0.06	0.52 ± 0.04	0.47 ± 0.03	0.87 ± 0.04
WRKY25	1.00 ± 0.06	1.19 ± 0.08	1.26 ± 0.07	1.37 ± 0.05	1.32 ± 0.14	1.00 ± 0.14	1.61 ± 0.12	1.52 ± 0.15	1.77 ± 0.26	0.83 ± 0.05
WRKY29	1.00 ± 0.07	1.17 ± 0.06	1.34 ± 0.09	1.64 ± 0.13	1.59 ± 0.26	1.00 ± 0.14	2.16 ± 0.16	2.32 ± 0.18	2.38 ± 0.12	0.92 ± 0.10
ZAT12	1.00 ± 0.17	0.78 ± 0.11	0.41 ± 0.07	0.46 ± 0.11	0.72 ± 0.14	1.00 ± 0.28	0.51 ± 0.10	0.27 ± 0.04	0.26 ± 0.06	0.72 ± 0.33

4.2.3.2. Exposure of Arabidopsis to excess Cu or Cd induces time-dependent and metal-specific changes in gene expression of antioxidative enzymes

When studying the gene transcripts of the antioxidative enzymes in roots (Table 4.2.), different responses to Cu and Cd were observed. Expression of the Cu/Zn superoxide dismutases *CSD1* and *CSD2* was already elevated after 2h exposure to Cu. In contrast, gene expression of both, *CSD1* and *CSD2,* was diminished at 24h in Cd-exposed plants. The gene expression of the Fe superoxide dismutase *FSD1* showed also different responses after exposure to Cu or Cd; Cu exposure reduced the *FSD1* expression from 6h exposure onwards, leaving almost no expression at 24h, while Cd caused increased *FSD1* transcript levels after 24h exposure. Gene expression of the ascorbate peroxidase *APX1* was induced at early time points after Cu exposure whereas Cd-exposed roots showed no differences in *APX1* transcript levels. When exposed to Cu, gene expression of catalase isozymes is differently regulated. Expression of *CAT1* is upregulated after Cu exposure, whereas *CAT2* and *CAT3* expression is inhibited. In case of Cd exposure, only *CAT1* showed a significant increase at 24h.

In leaves (Table 4.3.), expression of *CSD1* and *CSD2* was significantly upregulated after 6 and 24h exposure to Cu. Gene expression of *FSD1* was significantly reduced after 6 and 24h. In Cd-exposed plants, no significant differences in expression of these genes were observed. Gene expression of *APX1* was not altered by exposure to Cu or Cd. With regard to the catalases, *CAT3* showed an increased expression after 24h exposure to Cu. In leaves of plants exposed to Cd for 24h, *CAT2* transcript levels were reduced, while expression of *CAT3* was increased.

4.2.3.3. Exposure of Arabidopsis to excess Cu or Cd induces time-dependent alterations in gene expression of enzymes involved in ROS signalling

In order to gather more information concerning the signalling involved in stress responses in *A. thaliana* after metal stress, the gene expression of certain MAPK cascade components and transcription factors was measured.

In roots (Table 4.2.) of Cu-exposed *Arabidopsis* seedlings, gene expression of the protein kinase *OXI1*, MAPkinase *MPK3*, and transcription factors *WRKY22*, *WRKY25*, *WRKY29* and *ZAT12* showed a significant upregulation from the first measurement (2h) after the start of the exposure. After 24h, gene expression

levels were lower than the values observed during the first hours after the start of the exposure, but remained elevated in comparison to control plants. A transient increase in MAPkinase *MPK6* transcript level was observed after 6h Cu exposure. *ANP1* expression showed initially a reduction but was elevated after 6 and 24h exposure to Cu. Cadmium exposure led to a significant increase in gene expression of all kinase and transcription factor genes measured, except for *WRKY22*, and in contrast to Cu exposure this was observed only after 24h.

Table 4.2.: *Transcript levels of ROS-producing, antioxidative and signalling enzymes, and transcription factors relative to the non-exposed plants (= 1) at their respective time points, in roots of 3-week-old Arabidopsis thaliana plants exposed to 2 µM $CuSO_4$ or 5 µM $CdSO_4$ during 2, 4, 6 and 24h. Values are mean ± S.E. of at least 5 biologically independent replicates (significance levels: downregulation:* p < 0.05 | p<0.01 | p < 0.001 *; upregulation:* p < 0.05 | p<0.01 | p < 0.001 *).*

ROOTS								
	2h		4h		6h		24h	
Gene	Cu	Cd	Cu	Cd	Cu	Cd	Cu	Cd
LOX1	1.79±0.07	0.84±0.14	21.4±2.03	0.79±0.09	33.7±4.30	1.51±0.35	38.8±4.49	9.13±2.05
RBOHC	2.22±0.22	0.91±0.05	5.73±0.47	1.13±0.05	3.87±0.40	1.14±0.13	0.46±0.03	0.94±0.08
RBOHD	9.26±0.93	1.29±0.21	12.2±1.02	1.10±0.12	11.5±2.28	1.24±0.30	10.2±0.77	2.27±0.28
CSD1	1.67±0.06	0.98±0.06	2.02±0.14	1.10±0.05	2.17±0.09	0.85±0.04	2.90±0.22	0.47±0.04
CSD2	1.21±0.05	1.09±0.02	1.17±0.06	0.96±0.09	1.22±0.01	0.95±0.07	2.15±0.23	0.43±0.04
FSD1	0.92±0.20	1.19±0.22	0.83±0.12	0.88±0.10	0.32±0.06	1.30±0.29	0.012±0.005	4.24±0.33
APX1	1.28±0.07	0.92±0.02	1.73±0.10	0.92±0.03	1.41±0.14	0.85±0.04	1.17±0.06	0.92±0.04
CAT1	1.53±0.18	1.08±0.04	2.57±0.05	1.10±0.07	3.74±0.49	1.16±0.14	16.6±1.99	3.43±0.54
CAT2	0.33±0.03	1.02±0.10	0.35±0.02	0.89±0.02	0.43±0.02	0.81±0.05	0.34±0.07	0.79±0.04
CAT3	0.32±0.06	1.75±0.15	0.41±0.04	1.23±0.17	0.60±0.06	1.08±0.16	1.42±0.27	1.65±0.21
ANP1	0.63±0.03	1.12±0.06	1.34±0.09	1.34±0.07	1.86±0.12	1.19±0.07	2.01±0.19	1.32±0.06
OXI1	36.5±2.61	0.92±0.17	54.3±4.37	1.08±0.04	42.6±6.90	1.09±0.20	15.8±0.78	3.36±0.42
MPK3	6.78±0.73	1.23±0.15	3.39±0.21	1.17±0.11	2.22±0.08	1.38±0.17	2.78±0.29	2.56±0.18
MPK6	1.23±0.06	0.89±0.06	1.22±0.06	1.04±0.05	1.50±0.15	1.07±0.03	1.33±0.04	1.17±0.03
WRKY22	2.32±0.28	1.06±0.09	2.17±0.08	0.83±0.07	2.47±0.27	0.99±0.07	2.14±0.38	0.80±0.03
WRKY25	5.11±0.49	0.93±0.08	4.83±0.32	1.18±0.08	4.30±0.24	1.30±0.15	2.40±0.13	1.87±0.19
WRKY29	3.06±0.31	0.79±0.09	3.06±0.17	0.99±0.13	3.61±0.15	1.16±0.14	2.14±0.28	2.01±0.09
ZAT12	370±51	0.75±0.13	948±203	1.25±0.19	1447±201	1.24±0.25	88±11	10.2±3.01

Table 4.3.: *Transcript levels of ROS-producing, antioxidative and signalling enzymes, and transcription factors relative to the non-exposed plants (= 1) at their respective time points, in leaves of 3-week-old Arabidopsis thaliana plants exposed to 2 µM $CuSO_4$ or 5 µM $CdSO_4$ during 2, 4, 6 and 24h. Values are mean ± S.E. of at least 5 biologically independent replicates (significance levels: downregulation:* p < 0.05 | p<0.01 | p < 0.001 *; upregulation:* p < 0.05 | p<0.01 | p < 0.001 *).*

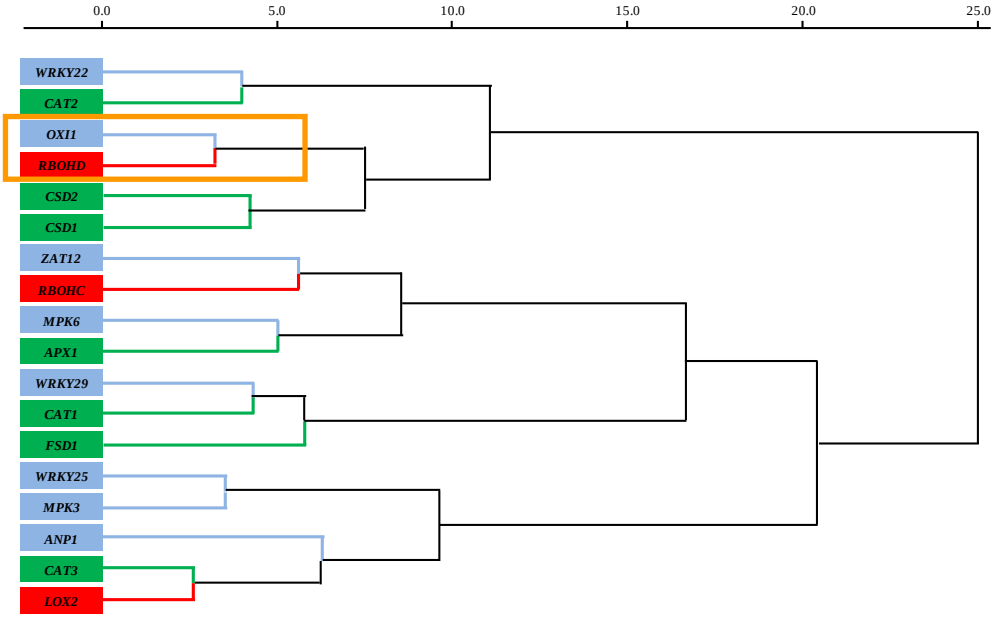
LEAVES								
	2h		4h		6h		24h	
Gene	Cu	Cd	Cu	Cd	Cu	Cd	Cu	Cd
LOX2	0.35±0.02	0.88±0.02	0.52±0.15	1.12±0.06	2.83±0.28	1.07±0.09	9.89±1.16	3.55±0.70
RBOHC	0.59±0.30	1.12±0.43	0.82±0.41	0.57±0.21	0.42±0.12	0.64±0.14	1.84±0.95	9.58±2.81
RBOHD	1.18±0.09	0.87±0.04	1.01±0.27	1.09±0.04	2.08±0.32	1.17±0.12	3.27±0.46	1.77±0.07
CSD1	1.03±0.05	0.89±0.06	0.81±0.11	1.06±0.14	1.44±0.12	0.87±0.12	4.38±0.16	0.81±0.16
CSD2	1.10±0.11	0.88±0.02	0.76±0.12	1.12±0.06	0.92±0.10	1.07±0.09	1.73±0.04	3.55±0.70
FSD1	0.69±0.13	1.25±0.21	0.76±0.13	0.80±0.21	0.42±0.06	1.48±0.35	0.023±0.003	1.03±0.18
APX1	0.93±0.10	0.97±0.05	0.84±0.04	0.92±0.03	1.02±0.09	0.94±0.14	1.18±0.03	1.13±0.07
CAT1	0.78±0.09	1.23±0.06	0.95±0.05	1.01±0.07	0.84±0.06	1.10±0.10	0.89±0.05	1.30±0.13
CAT2	0.87±0.06	0.96±0.06	0.74±0.05	0.96±0.06	0.70±0.04	1.03±0.06	0.81±0.06	0.79±0.04
CAT3	0.68±0.07	1.14±0.05	0.80±0.08	1.02±0.03	1.22±0.12	1.43±0.30	2.80±0.35	1.48±0.21
ANP1	0.83±0.09	0.85±0.03	1.01±0.06	1.04±0.05	0.98±0.07	1.00±0.05	1.32±0.05	1.41±0.08
OXI1	3.12±0.81	0.78±0.10	1.21±0.55	0.92±0.16	1.79±0.38	0.63±0.07	3.46±0.67	21.3±2.83
MPK3	2.22±0.36	1.02±0.06	1.51±0.13	1.12±0.10	1.46±0.08	1.29±0.06	2.37±0.14	2.68±0.42
MPK6	0.93±0.08	0.92±0.04	0.85±0.10	0.98±0.03	1.08±0.11	1.07±0.01	1.32±0.12	1.27±0.12
WRKY22	3.08±0.36	0.89±0.05	0.53±0.06	0.92±0.07	0.67±0.03	0.83±0.04	0.96±0.06	1.00±0.05
WRKY25	2.17±0.42	1.08±0.10	1.18±0.14	1.04±0.16	1.41±0.19	1.30±0.03	2.25±0.17	5.72±0.61
WRKY29	1.08±0.19	0.83±0.03	0.74±0.07	0.82±0.06	0.63±0.07	0.96±0.04	0.68±0.09	1.14±0.04
ZAT12	0.49±0.33	0.42±0.05	1.25±0.69	0.95±0.31	1.52±0.32	1.99±0.64	4.47±1.11	18.3±3.64

In leaves (Table 4.3.), the expression of some signalling genes showed a biphasic response in plants exposed to Cu. The *OXI1* expression was elevated after 2h of exposure, returned to the control level after 4h and increased again after 24h. With regard to the downstream MAPKinases, *MPK3* showed a significant increase after 2 and 24h exposure to Cu similarly to *OXI1*, whereas gene expression of *MPK6* did not change after exposure to Cu. Gene expression of the transcription factor *WRKY25* was also elevated after 2 and 24h exposure to Cu. An immediate, but transient rise in the transcript level of *WRKY22* was noticed in Cu-exposed plants, whereas a decreasing trend of *WRKY29* expression was observed at 4, 6 and 24h exposure to Cu. After Cd exposure, the *OXI1*

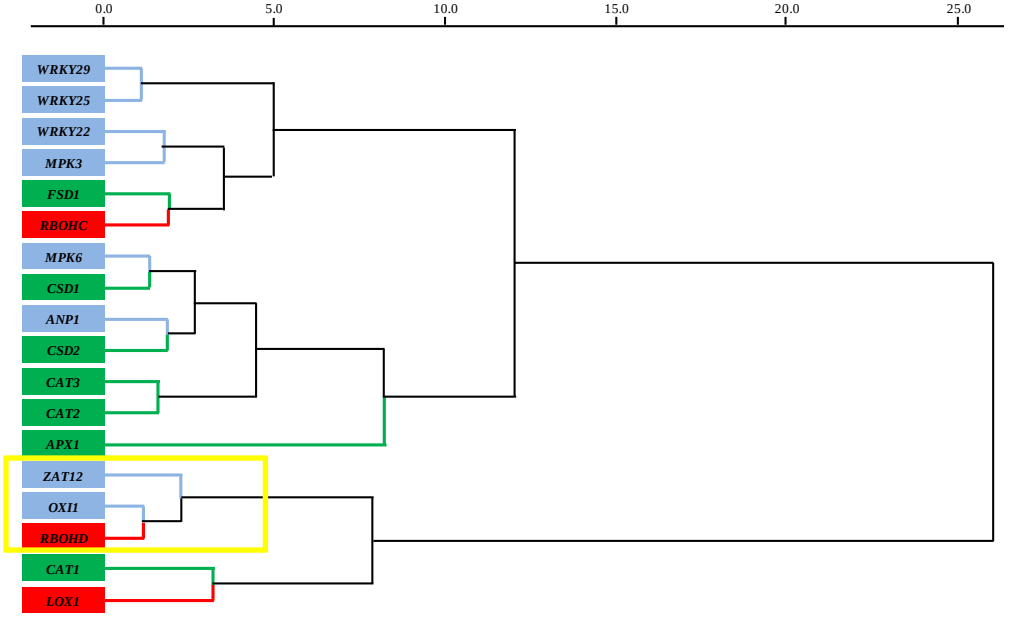
expression was significantly increased after 24h concomitant with an increased gene expression of *MPK3* and *MPK6*. Whereas no induction of *WRKY22/*29 was observed in Cd-exposed plants, *WRKY25* and *ZAT12* showed a significant induction in gene expression after 24h.

Gene clustering analysis using GenEx Software, which searches for similar expression patterns or more specific coregulation of genes, was performed on the gene expression results of Cu- and Cd-exposed roots and leaves (Figure 4.3.). Genes placed close together in a cluster, are predicted to have a similar gene expression pattern. A predicted cluster, seen in Cu- and Cd-exposed roots as well as in Cd-exposed leaves, was the cluster between *OXI1*, *ZAT12* and *RBOHD*. In Cu-exposed leaves, *OXI1* formed a cluster with only *RBOHD* while *ZAT12* seems to be clustered to *RBOHC*. Other clusters that are worth mentioning are the *CSD1/CSD2* and the *WRKY25/MPK3* clusters seen in Cu- and Cd-exposed roots and leaves.

A



B


0.0
5.0
10.0
15.0
20.0
25.0
WRKY29
WRKY25
WRKY22
MPK3
FSD1
RBOHC
MPK6
CSD1
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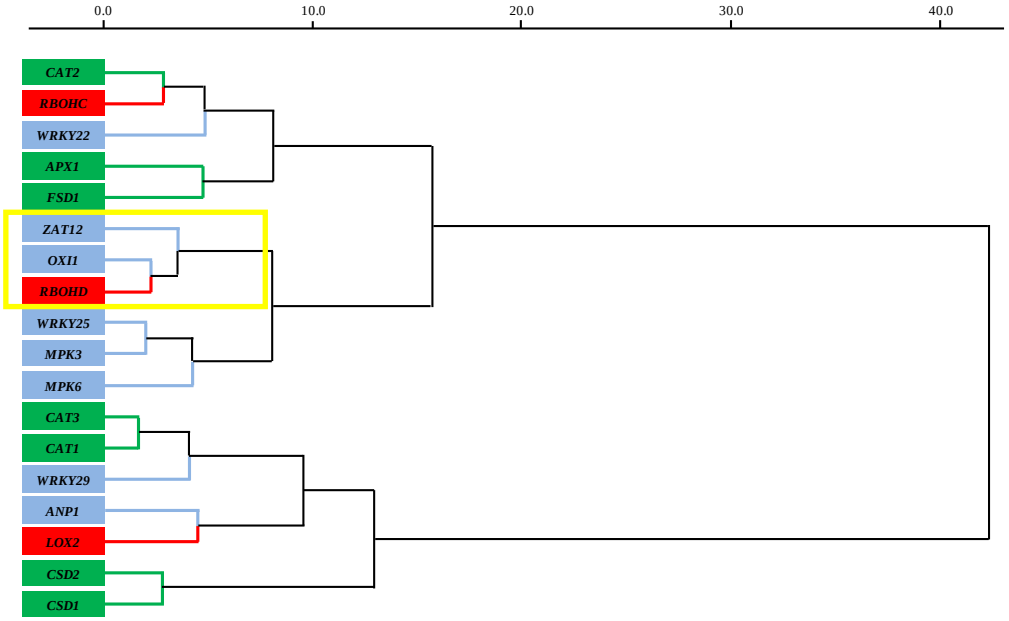
CSD2
CAT3
CAT2
APX1
ZAT12
OXI1
RBOHD
CAT1
LOX1

C


0.0
10.0
20.0
30.0
40.0
CAT2
RBOHC
WRKY22
APX1
FSD1
ZAT12
OXI1
RBOHD
WRKY25
MPK3
MPK6
CAT3
CAT1
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ANP1
LOX2
CSD2
CSD1

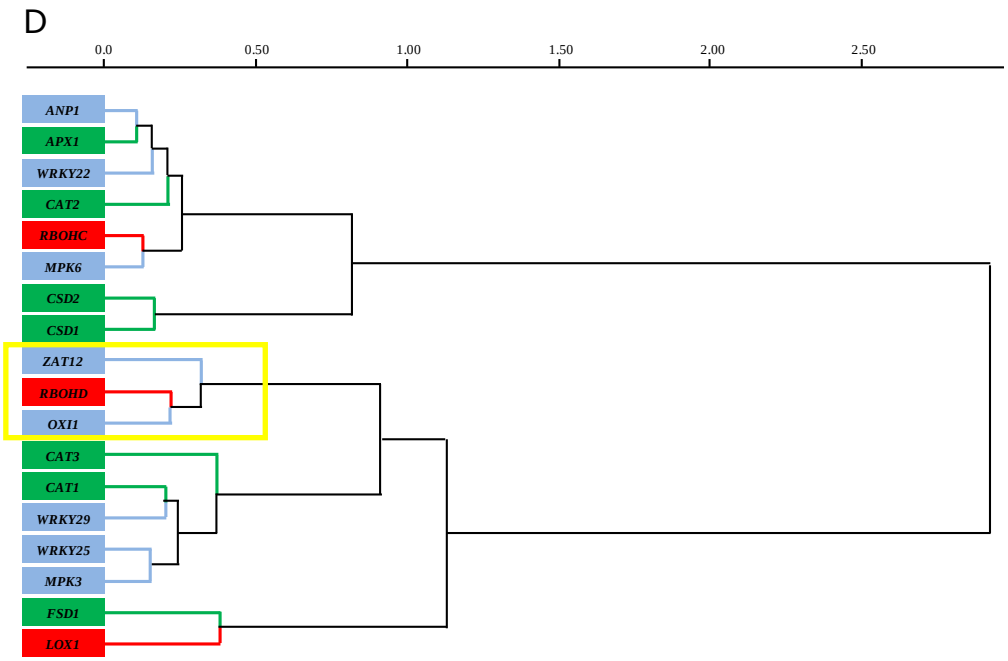

Figure 4.3.: *Gene expression clusters predicting possible coregulation of genes in respectively Cu and Cd-exposed leaves* ***(A-C)*** *and roots* ***(B-D)****. Red, green and blue boxes indicate respectively pro-oxidative, antioxidative or signalling genes.*

4. Discussion

Exposure of plants to toxic concentrations of Cd and Cu leads to physiological defects like disturbances in growth (Cuypers *et al*. 2009). In our experiment, the immediate effect of Cu on growth caused already after 24h an almost 50% difference in root fresh weight as compared to control plants (Figure 4.1.). However, dry root weight didn't differ between control and Cu-exposed plants. Therefore, the reduction in root fresh weight after Cu exposure seems to be caused by water loss from the root rather than by alterations in root growth processes. In contrast, 24h exposure to Cd did not have any effect on leaf or root weight (Figure 4.1.). These responses are in accordance with earlier results on *Arabidopsis thaliana* (Smeets *et al*. 2008a, 2009).

At the cellular level, exposure of plants to elevated Cu and Cd concentrations induces oxidative stress (Smeets *et al*. 2005, Cuypers *et al*. 2011a). Metal-induced ROS production can be caused by Fenton and Haber-Weiss reactions (Kehrer 2000) or by activation of ROS-producing enzymes, like NADPH oxidases (Figure 4.4.) (Olmos *et al*. 2003, Garnier *et al*. 2006, Heyno *et al*. 2008). ROS

can then activate MAPK cascades, like OXI1-MPK3/6 (Figure 4.4.). MAPK cascades are responsible for the regulation of transcription factors (WRKY22/25/29, ZAT12), which can modulate antioxidative responses of the plant to metal stress (Figure 4.4.). In this study, responses of known signalling components were examined after disturbance of the redox balance by short-term exposure to low concentrations of Cu and Cd, using real-time PCR. Here, it is important to mention that leaf transcript levels of non-exposed *Arabidopsis* plants varied significantly in time for almost all genes investigated in this study (Table 4.1.). This knowledge is particularly useful when interpreting results of gene expression analysis. Consequently, a so-called up- or downregulation in gene expression after metal exposure can be either due to time-dependent variations in gene expression, rather than to metal-induced changes. Therefore, it is essential to investigate control samples alongside samples from exposed plants at all time points, and to express the results of metal-exposed plants relative to the gene expression levels in non-exposed plants at the same time point.

The disturbance of the cellular redox balance after Cu exposure is reflected by the increased H_2O_2 levels in the roots (Figure 4.2.A). This corresponds to the immediate Cu-induced upregulation of the gene expression of ROS-producing NADPH oxidases (*RBOHC* and *D*), while at the same time gene expression of the different CAT-isoforms after Cu exposure seems to be balanced (upregulation of *CAT1*, downregulation of *CAT2/3*) (Table 4.2). In addition, Cuypers *et al*. (2011a) showed that the activity of catalase is not changed in roots of *A. thaliana* after 24h exposure to 2 µM Cu. Consequently, the produced H_2O_2 cannot be neutralized by catalases and is available for Haber-Weiss and Fenton reactions. In this way H_2O_2 formed by the dismutation of $O_2^{\bullet -}$, produced by NADPH oxidases (Torres and Dangl 2005), can be converted into the more reactive hydroxyl radical causing membrane damage, that is reflected in the immediate lipid peroxidation (Figure 4.2.B) occurring in Cu-exposed roots (Figure 4.4.). In leaves of *Arabidopsis* plants exposed to either Cu or Cd, no significant changes in H_2O_2 content were found, although an increasing trend was seen for both metals after 24h exposure. The difference in H_2O_2 response between Cd- or Cu- exposed roots was earlier seen in roots of alfalfa seedlings exposed to Cd or Hg (Ortega-Villasante *et al*. 2007), and suggests that redox-

active metals trigger ROS production faster than non redox-active. However in other plant species, increases of the H_2O_2 content in roots were seen at 48h after the start of Cu exposure in *Lemna minor* (Panda 2008) and already 6h after the onset of Cd exposure in *Pinus sylvestris* (Schützendübel *et al*. 2001). It is clear that in order to make comparisons between plant responses to different metals, similar experimental conditions should be used. In leaves of *Arabidopsis* and other plant species exposed to Cu and Cd, increases in H_2O_2 content have also been observed (*Arabidopsis*: Maksymiec and Krupa (2006); *Pisum sativum*: Rodriguez-Serrano *et al*. (2009); *Plagomnium cuspidatum*: Wu *et al*. (2009)). However, the concentrations of Cd and Cu and/or the exposure times used in these studies were much higher/longer in comparison to our study.

Whereas lipid peroxidation might be a direct consequence of Cu-induced ROS production, lipoxygenases can also initiate this process (Figure 4.4.). Lipoxygenases are enzymes that catalyze oxygenation of polyunsaturated fatty acids into lipid hydroperoxides, which are involved in responses to different stresses (Skorzynska-Polit *et al*. 2006). In our study, this is reflected in the elevated *LOX1* and *LOX2* gene expression after Cu exposure (Table 4.2. and 4.3). Cd-exposed plants did not show any changes in lipid peroxidation, although lipoxygenase transcript levels were upregulated after 24h Cd exposure. Earlier work in our group reported an increase in lipid peroxidation in roots of *Arabidopsis* seedlings after 24h exposure to 10 µM Cd (Smeets *et al*. 2009, Cuypers *et al*. 2011a). The changes in lipoxygenase gene expression that did not lead to measurable membrane damage may be involved in altered oxylipin signalling after exposure of plants to Cd or excess Cu (Remans *et al*. 2010).

To cope with these disturbances in cellular redox state, the antioxidant defence system was induced in the roots of *Arabidopsis* seedlings immediately after Cu exposure (Table 4.2.), whereas responses were only observed after 24h in case of Cd exposure. This can be explained by the different redox behaviour of both metals, with a delay in indirect induction of ROS by Cd compared to direct ROS production by Cu. To activate gene expression of ROS-producing or antioxidative enzymes, signalling via MAPkinases or other signalling compounds within the plant cell is needed (Figure 4.4.) (Mittler *et al*. 2004). For this purpose, gene expression of protein kinases (OXI1, ANP1, MPK3, MPK6) and transcription factors (WRKY22, WRKY25, WRKY29, ZAT12), known to play a role in abiotic

stress responses (Suzuki *et al*. 1999, Kovtun *et al*. 2000, Asai *et al*. 2002, Rentel *et al*. 2004, Davletova *et al*. 2005a,b, Zheng *et al*. 2007, Li *et al*. 2009), was measured in *Arabidopsis* plants after exposure to Cu and Cd.

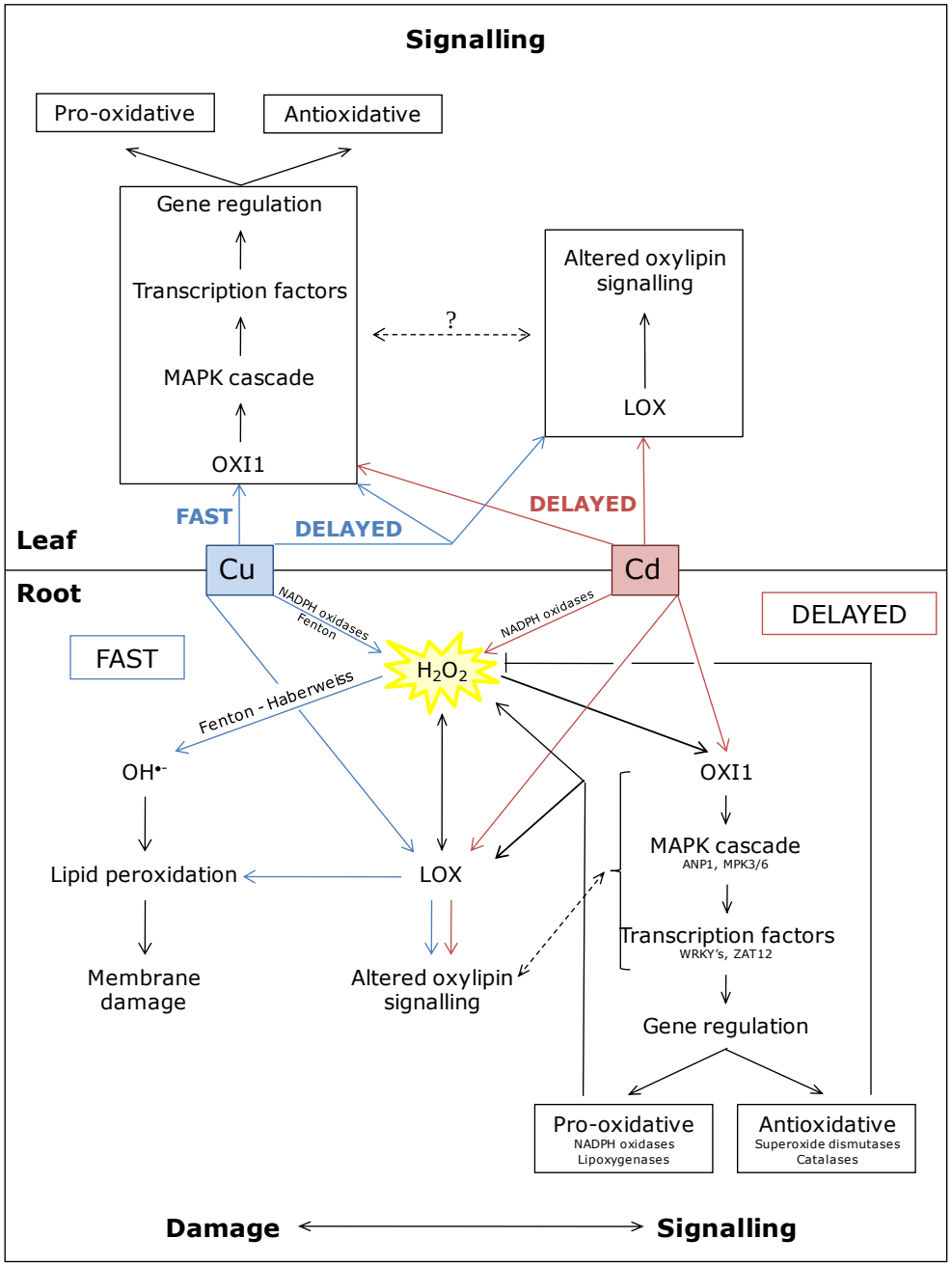

Figure 4.4.: *Overview of ROS signalling in metal stress. In Cu-exposed roots, H_2O_2 can be produced via an induction of NADPH oxidases or Fenton reactions. H_2O_2 can act as a signalling molecule, thereby activating the OXI1-MAPK cascade leading to gene regulation of pro- and antioxidative enzymes. The balance between these pro- and antioxidants and the specific signalling cascades induced determine the outcome of this pathway. On the other hand, H_2O_2 can give rise to harmful hydroxyl radicals ($OH^{\bullet}$), which induce lipid peroxidation of cell membranes, leading to irreversible membrane damage, a condition that can be enhanced by the Cu-induced lipoxygenase (LOX) activity. Cd exposure of roots leads only after 24h exposure to an induction of NADPH oxidases (RBOHD) and consequently a slight increase in H_2O_2 production followed by the delayed induction of gene regulation via MAPK signalling. Although Cd exposure induces lipoxygenases, no lipid peroxidation is seen suggesting an interaction between lipoxygenases and MAPK cascades via an altered oxylipin signalling. In the leaves of Cu- and Cd-exposed plants, signalling via OXI1-MAPK cascades and LOX is induced in a time-dependent manner. Cu is responsible for a fast (2h) as well as a delayed (24h) induction of the OXI1-cascade with the delayed induction possibly induced by oxylipin signalling.*

In roots, expression of most of these genes is rapidly upregulated after the start of exposure to Cu. Once again, in case of Cd, changes in gene expression were not detected before 24h of exposure. The highest responses in gene expression of *Arabidopsis* roots after Cu exposure were observed for *OXI1* and *ZAT12*. Gene clustering analysis predicted a clustering between *OXI1* and *ZAT12* in roots of Cu-exposed plants (Figure 4.3.). The similar regulation of gene expression of *OXI1* and *ZAT12* could be explained by the fact that both proteins are activated by H_2O_2 produced during oxidative stress and probably their gene expression could be regulated in the same way (Rentel *et al*. 2004, Davletova *et al*. 2005b). Indeed in our study, Cu-induced expression of *OXI1* and *ZAT12* seems to correspond to an increase in H_2O_2-production. According to the gene clustering analysis, *OXI1* and *ZAT12* also clustered together with *RBOHD* in Cu- and Cd-exposed roots and even in Cd-exposed leaves. A clustering of *OXI1* and *RBOHD* was also seen in leaves of Cu-exposed plants (Figure 4.4.). So expression of these genes seems also to be coordinately regulated during metal stress responses in *A. thaliana*. Also in case of plant-pathogen interactions, a relation between RBOHD and *OXI1* has been suggested (Petersen *et al*. 2009). Davletova *et al*. (2005a) suggested that MAPK-dependent regulation of NAPDH oxidase (RBOHD) expression might be involved in amplification of the ROS signal; Miller *et al*. (2009) indeed showed that RBOHD mediates systemic signalling in response to abiotic stress. Therefore, increased RBOHD expression may be important in signalling metal stress from roots to leaves. The production of ROS by NADPH oxidases and more specifically RBOHD would drive the expression of *OXI1*. Takahashi *et al*. (2011) were able to demonstrate a

relationship between the *RBOHD* regulated *OXI1* and *ZAT12* expression and MAPK signalling. In the case of wounding, they showed that the MAPK-cascade MKK3-MPK8 negatively regulates the gene expression of *OXI1* and *ZAT12* via negative regulation of *RBOHD*. Also important to mention is the expression of *WRKY22,* which is upregulated after exposure to Cu, but unchanged after exposure to Cd. Little is known about this transcription factor, except that, like other WRKY transcription factors, it binds TGAC core sequences in the promoters of many defence-related genes (Pandey and Somssich 2009) and that it is induced by MPK3 and MPK6 (Asai *et al*. 2002). Investigation of the promoters of the gene cluster *RBOHD/OXI1/ZAT12* for cis-acting elements using the PLACE and PlantCARE databases (Prestridge 1991, Higo *et al*. 1999, Lescot *et al*. 2002), revealed that the WRKY-binding site was detected very often in these promoters, suggesting a possible role for WRKY transcription factors in the regulation of *RBOHD*, *OXI1* and *ZAT12* genes. In addition to that, our results suggest that *WRKY22* is a metal-specific transcription factor as it is involved in Cu responses, but not in Cd responses.

Possible targets of gene regulation by MAPK cascades are genes involved in maintaining the cellular redox status (Figure 4.4.). Gene expression of the catalase isozymes *CAT1*, *CAT2* and *CAT3* is differently regulated in roots after Cu exposure (Table 4.2.). The *CAT1* expression is upregulated after Cu exposure, whereas the gene expression of *CAT2* and *CAT3* is downregulated. Xing *et al*. (2007, 2008) showed that stress-induced gene expression of the *Arabidopsis* catalase family consists of two major signalling pathways; one AtMEK1/MPK6- and H_2O_2-mediated pathway leading to *CAT1* expression and another unknown pathway leading to *CAT2* and *CAT3* expression. Possibly, increased *CAT1* gene expression in roots after Cu exposure is due to the induction of the H_2O_2-mediated pathway. Furthermore, also *APX1* transcript levels are elevated immediately after Cu exposure. According to Davletova *et al*. (2005a,b), enhanced expression of *APX1* is induced by the transcription factor ZAT12 in response to ROS production. Concerning the very low gene expression of ZAT12 under non-exposed conditions and the strong increase after Cu exposure, it can be suggested that ZAT12 is a stress-responsive transcription factor, which via APX1 plays a central role in oxidative stress responses induced by Cu. Moreover, expression of the Cu/Zn superoxide dismutase genes *CSD1* and *CSD2* was

induced in roots after exposure to Cu, whereas their gene expression was inhibited after Cd exposure (Table 4.2.). Sunkar *et al*. (2006, 2007) revealed a mechanism of posttranscriptional gene regulation via miRNA398-mediated mRNA degradation and translational repression for *CSD1* and *CSD2*. Like Cuypers *et al*. (2011a), they demonstrated that short-term exposure to Cu led to decreased *miRNA398* expression levels, which caused an accumulation of *CSD1* and *CSD2* transcripts. Cuypers *et al*. (2011a) also showed that the opposite was seen for Cd-exposed plants. Furthermore, Yamasaki *et al*. (2009) reported that SPL7 is essential for the response of miRNA398 to Cu. Under Cu deficiency, SPL7 is able to bind to GTAC motifs in the promoter of miRNA398 thereby activating their transcription and allowing them to reduce the expression of *CSD* transcripts. In the case of Cu excess, a decrease of *miRNA398* and an induction of *CSD* transcript levels was observed. A possible involvement of SPL7 might be also responsible for the different regulation of *FSD1* after Cu and Cd exposure in roots (Table 4.2.) (Nagae *et al*. 2008, Cuypers *et al*. 2011a). Although *CSD1* and *2* after Cu exposure showed similar expression patterns as the MAPkinase genes and transcription factors, no connection between MAPkinase signalling and gene regulation of superoxide dismutases is known to date. Genes involved in the antioxidative defence of the cell could be regulated by MAPK cascades, and MAPK cascades could also regulate the gene expression of pro-oxidative enzymes, which in turn are involved in amplification of the ROS signal in the cell (Figure 4.4.). Examples are the elevated transcript levels of *LOX1/2* and *RBOHC/D* after Cu and Cd exposure. Petersen *et al*. (2009) described a relation between RBOHD and *OXI1* expression in case of plant-pathogen interactions and RBOHD is involved in amplification of the ROS signal in response to abiotic stress (Miller *et al*. 2009).

In conclusion, Cd and Cu cause different responses in *Arabidopsis thaliana* seedlings. Copper altered H_2O_2 levels, lipid peroxidation and gene expression of enzymes involved in ROS production, antioxidative defence and signalling, already after 2h of exposure to environmental realistic concentrations. *Arabidopsis* seedlings exposed to Cd showed changes in these parameters only after 24h exposure. The fast effects may be related to the redox-active properties of Cu, which enable direct ROS production causing the immediate

onset of antioxidative responses as well as damage to cellular molecules and structures. In contrast, the delayed response to Cd may be due to indirect ROS production, which first requires interaction with the functional groups of enzymes and/or substitution of their metal cofactor to inhibit their function. Furthermore, we found metal specific regulation of pro-oxidative genes (*e.g. RBOHC*), antioxidative genes (*e.g.* superoxide dismutase genes) and transcription factors (*WRKY22*). This suggests that Cd and Cu induce specific ROS and MAPK signalling cascades due to different chemical properties and/or perception of these metals.

Chapter 5

The protein kinase OXI1 mediates Cu-induced plant responses in *Arabidopsis thaliana*

Kelly Opdenakker, Tony Remans, Ann Ruttens, Celine Forzani, Heribert Hirt, Jaco Vangronsveld, Ann Cuypers (2012). The protein kinase OXI1 mediates Cu-induced plant responses in *Arabidopsis thaliana*. Submitted.

Abstract

In this study, we investigated the role of *Oxidative signal-inducible 1* (OXI1) in the oxidative stress response in *Arabidopsis thaliana* plants exposed to excess of the redox-active element copper (Cu). OXI1 is a serine/threonine protein kinase, known to activate the mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 in response to reactive oxygen species (ROS). MAPKs are able to regulate transcription of target genes by the phosphorylation and subsequent activation of transcription factors. Our findings show that OXI1 plays a role in the regulation of Cu uptake and translocation in Cu-exposed *Arabidopsis* plants. In accordance with the retention of the redox-active Cu in the roots, more membrane damage was observed in roots of Cu-exposed wild type plants. Furthermore, a strong decrease in transcript levels of the jasmonate (JA) responsive *PDF1*.2 gene was observed in leaves of control and Cu-exposed plants demonstrating the involvement of OXI1 in Cu-induced oxylipin signalling. After long-term Cu exposure, these OXI1-regulated stress responses can result in wild type plants that are more susceptible to excess Cu than *oxi1* knockout plants.

5.1. Introduction

Copper (Cu) is an essential trace element for the plant. However, in case of exposure to increased concentrations, Cu can become toxic and cause stress effects in plants (Cuypers *et al*. 2009, Yruela 2009). Because Cu is a redox-active element, it is able to generate reactive oxygen species (ROS) directly via Fenton and Haber-Weiss reactions (Kehrer 2000) and therefore can induce oxidative stress at the cellular level (Smeets *et al*. 2009, Cuypers *et al*. 2011a). Although the rate of ROS production is elevated during stress conditions, low levels of ROS are essential for normal cell metabolism. This requires fine-tuning of subcellular ROS levels by the antioxidative defence system of the cell, consisting of enzymes and metabolites (Mittler *et al*. 2004, Halliwell 2006).

As signalling molecules, ROS play a key role in processes mediating defence against biotic and abiotic stresses (Torres and Dangl 2005) but also during normal cell growth and development (Foreman *et al*. 2003). For example, H_2O_2 can modulate the activities of many components in cell signalling, such as protein phosphatases, protein kinases and transcription factors (Miller *et al*. 2008, Quan *et al*. 2008). One of them is the serine/threonine kinase *Oxidative signal-inducible 1* (OXI1), also known as AGC2-1 because OXI1 is a member of the AGC protein kinase family. OXI1 was first identified as a downstream signalling component of the *3-phosphinositide-dependent protein kinase 1* (PDK1) in phosphatidic acid (PA) signalling (Anthony *et al*. 2004). Later, a function as protein kinase required for oxidative burst-mediated signalling involved in root hair growth and basal resistance to *Peronospora parasitica* infection, was discovered (Rentel *et al*. 2004). Petersen *et al*. (2009) proposed that the expression and activation of OXI1, in case of oxidative burst-mediated signalling, is driven by ROS produced through NADPH oxidases and confers tolerance to *Pseudomonas syringae* infection. Together, OXI1 and its downstream target, the serine/threonine kinase PTI1-2, are capable of integrating lipid-derived and ROS stress signals (Anthony *et al*. 2006). Anthony *et al*. (2006) also demonstrated that the expression of ROS promoters was increased after activation of PTI1-2 by PA, H_2O_2, the fungal elicitor xylanase and flagellin. This may be possible via the involvement of mitogen-activated protein kinases (MAPK) cascades since MAPK cascades can regulate gene transcription by activation or repression of transcription factors (Colcombet and Hirt 2008).

Rentel *et al*. (2004) reported that OXI1 is required for the full activation of the MAPKs, MPK3 and MPK6. Recently, Camehl *et al*. (2011) showed that *Piriformospora indica* promotes plant growth in *Arabidopsis* via PA-stimulated activation of the PDK1/OXI1/MPK6 pathway. At the same time, Forzani *et al*. (2011) found that, in case of oxidative stress, wounding or cellulose treatment, OXI1 can activate MPK6 via the action of PTI1-4, another member of the PTI1-like family.

Although the role of the OXI1 kinase is clearly described in biotic stresses, involvement of OXI1 in abiotic stresses and especially metal stress is less well understood. In earlier work we showed a fast (2h) induction of OXI1 and the downstream kinase MPK3 after Cu exposure at the gene expression level (Opdenakker *et al*. 2012a; chapter 4). After Cd exposure, the same but delayed response was observed. Further, Smeets *et al*. (personal communication) suggest a major role for OXI1 in Cu-induced oxidative (defence) signalling since they reported a decrease in transcript levels of MAPKs, other regulatory proteins and antioxidative enzymes in roots of Cu-exposed *oxi1* mutant *Arabidopsis thaliana* plants (Ws ecotype).

Based on the findings that (1) OXI1 is activated by ROS and (2) its gene expression was induced under metal stress conditions, it was hypothesized that OXI1 is involved in mediating Cu stress responses. Therefore in this study, the role of OXI1 in Cu-induced stress signalling was further examined using OXI1 knockout *Arabidopsis thaliana* (Columbia-0 ecotype) seedlings grown in hydroponics as defined in earlier studies (Opdenakker et al. 2012a: chapter 4, Cuypers et al. 2011, Smeets et al. 2009). First, it was examined if the Cu content of leaves and roots was altered in *oxi1* mutant seedlings. Secondly, it was investigated whether OXI1 induces alterations in the cellular redox state or in oxidative stress signalling. Finally, it was tested if OXI1-mediated changes in Cu stress responses are involved in Cu sensitivity after long-term Cu exposure.

5.2. Results

5.2.1. OXI1 induces a Cu-specific phenotype

Three-week-old seedlings of *Arabidopsis thaliana*, grown on hydroponics, were exposed to 2 µM $CuSO_4$ during a short period (24h) and leaf and root fresh

weight were compared between wild type and *oxi1* mutant plants (Figure 5.1.). In the leaves, exposure to Cu reduced rosette weight in wild types significantly after 24h but had no effect on leaves of *oxi1* plants. Root weight was significantly decreased in wild type (7.95 ± 0.47 vs 19.68 ± 1.52 mg) as well as in *oxi1* plants (12.70 ± 1.28 vs 24.70 ± 2.71 mg) after 24h exposure to Cu, although root weight was less inhibited in *oxi1* plants (48% vs 60% in wild type plants).

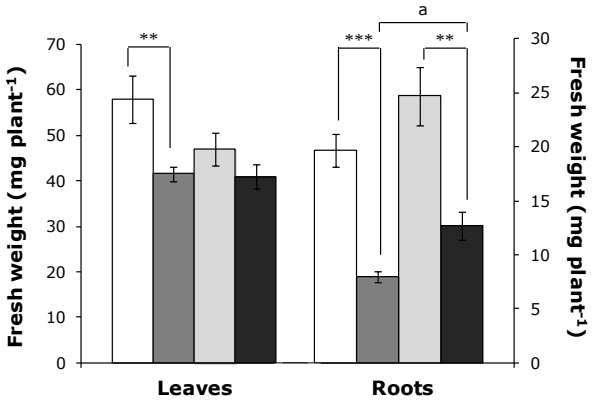

Figure 5.1.: *Leaf and root weight (mg plant^{-1}) of 3-week-old Arabidopsis thaliana wild type and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 24h (□ = Col-0 non-exposed, ■ = Col-0 exposed, □ = oxi1 non-exposed, ■ = oxi1 exposed). Values are mean ± S.E. of ± 20 biologically independent replicates (significance level: treatment effect: **: p < 0.05, ***: p < 0.01; interaction effect: a: p < 0.01). WT: wild type.*

5.2.2. OXI1 prevents translocation of Cu from root to shoot

To investigate if the OXI1-mediated Cu-specific phenotype is the result of alterations in the Cu content of leaves and roots, Cu concentrations of leaves and roots were analyzed in both wild type and *oxi1* mutant plants (Figure 5.2.A and 5.2.B). Leaves of wild type plants showed no difference in Cu content after 24h exposure to Cu while the leaves of *oxi1* plants contained significantly more Cu after exposure than control plants (Figure 5.2.A). In roots, the Cu content was significantly increased in both wild type and *oxi1* plants after Cu exposure (Figure 5.2.B), although the concentration of Cu was higher in wild type seedlings (1911.6 ± 391.2 vs 18.7 ± 4.9 mg kgDW^{-1}) as compared to *oxi1* mutant plants (914.2 ± 69.8 vs 12.5 ± 1.3 mg kgDW^{-1}). When the root-to-shoot translocation (shoot/root ratio) was calculated for wild type and *oxi1* plants, it appeared that Cu-exposed wild type plants (0.43 ± 0.02%) had a significant lower root-to-shoot translocation (p-value < 0.0001) than Cu-exposed *oxi1* mutants (1.31 ± 0.05%).

Expressions of genes involved in synthesis of metallothioneins (MTs) and phytochelatins (PC synthases) were determined to examine if the mutation of *OXI1* influenced transcript levels of these metal-sequestering low molecular weight polypeptides that could be responsible for the different Cu translocation observed in *oxi1* plants (Figure 5.2.C and D; Supplemental Tables 5.1. and 5.2.).

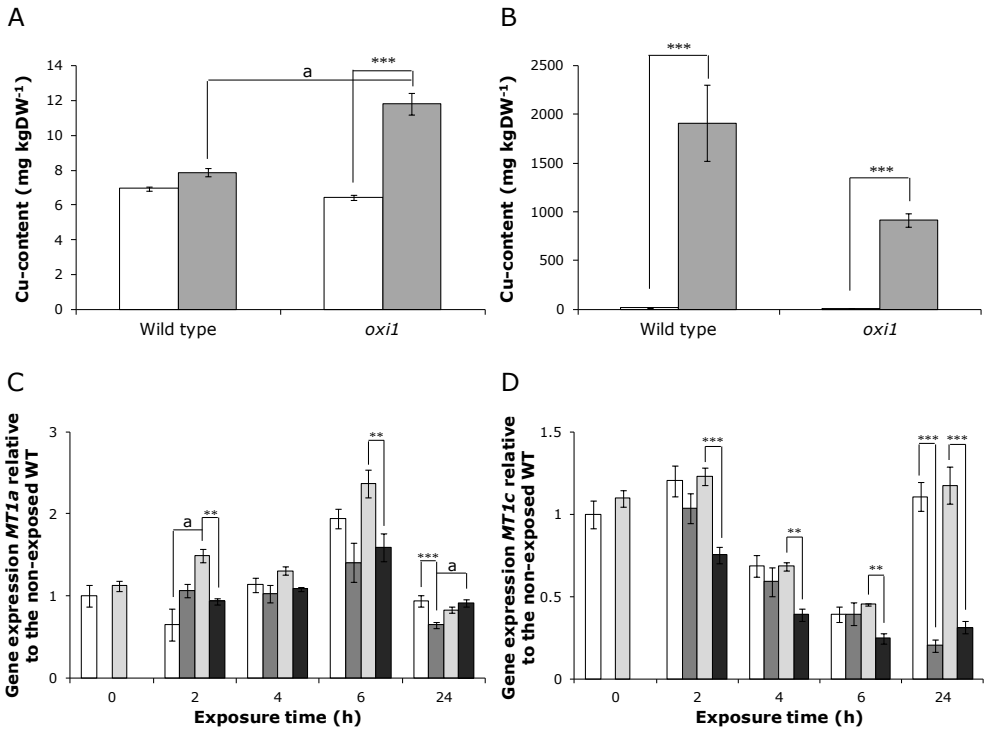

Figure 5.2.: (A) *and* ***(B):*** *Cu-content (mg kgDW^{-1}) of respectively leaves and roots of 3-week-old Arabidopsis thaliana wild type (WT) and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 24h (white bars = non-exposed plants; grey bars = exposed plants). Values are mean ± S.E. of ± 5 biological independent replicates (significance level: treatment effect: ***: p < 0.01; genotype effect: a: p < 0.01).* ***(C)*** *and* ***(D):*** *Transcript levels of respectively MT1a and MT1c in leaves of 3-week-old Arabidopsis thaliana wild type and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 2, 4, 6 and 24h (□= wild type non-exposed, ■= wild type exposed, □= oxi1 non-exposed, ■= oxi1 exposed). Gene expression was calculated relative to the non-exposed wild type plants (= 1). Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: treatment effect: **: p < 0.05; ***: p < 0.01; interaction effect: a: p < 0.01).*

MT1a expression in the leaves of *oxi1* mutant plants was transiently reduced after 2, 4 and 6h Cu exposure as compared to *oxi1* control plants, which was not observed in wild type plants (Figure 5.2.C). In wild type plants, *MT1a* transcript

levels were decreased after 24h exposure to Cu while the expression of *oxi1* plants returned to control levels. Whereas *MT1a* transcript levels showed a diurnal increase in leaves of control plants (Figure 5.2.C), gene expression of the other isoform metallothionein 1, *MT1c*, was downregulated during the day in the leaves of both genotypes (Figure 5.2.D). In addition, *MT1c* gene transcript levels further decreased in *oxi1* mutant seedlings already after 2h Cu exposure (Figure 5.2.D). Wild type plants showed only a decrease in *MT1c* expression levels after 24h exposure to Cu. Transcript levels of *PCS1* in the leaves were elevated after 2h Cu exposure in *oxi1* plants while it was increased after 6h Cu exposure in wild type plants (Supplemental Table 5.1.). In the roots, no differential gene expression was observed between wild type and *oxi1* mutant plants for *PCS1* or *MT* genes (Supplemental Table 5.2.).

5.2.3. OXI1 is involved in ROS-induced membrane damage

Since Cu is a redox-active element, alterations in Cu content of leaves and roots may be correlated to an altered ROS production. Therefore, lipid peroxidation, as a measure of ROS-induced cellular damage, was determined in leaves and roots of wild type and *oxi1* mutant seedlings after exposure to Cu. In leaves of wild type as well as *oxi1* plants, no significant differences in lipid peroxidation were detected after exposure to Cu (data not shown).

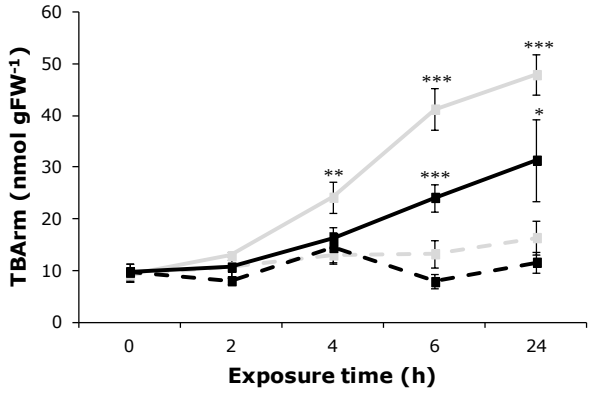

Figure 5.3.: *Lipid peroxidation (nmol gFW^{-1}) in roots of Arabidopsis thaliana wild type and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 0, 2, 4, 6 and 24h (grey dashed line = wild type non-exposed, grey line = wild type exposed, black dashed line = oxi1 non-exposed, black line = oxi1 exposed). Values are mean ± S.E. of ± 7 biologically independent replicates (significance level: *: p < 0.1; **: p < 0.05; ***: p < 0.01).*

In roots of wild type plants, the concentration of TBA reactive metabolites (TBArm) was already significantly higher after 4h of Cu exposure and further increased until 24h exposure to Cu (Figure 5.3.). In contrast, lipid peroxidation

in roots of *oxi1* mutant plants showed no significant increase before 6h of Cu exposure (Figure 5.3.). When the lipid peroxidation over all time points was considered together for respectively wild type and *oxi1* mutant seedlings, the observed increase in TBArm content of exposed *oxi1* roots was lower than for roots of wild type plants ($p = 0.0510$).

5.2.4. OXI1 regulates transcription of oxidative stress and signal transduction related genes

Because OXI1 is a signalling molecule, known to be involved in responses to oxidative stress and able to induce MAPK cascades, it was investigated if OXI1 can induce alterations in the gene expression of pro- or antioxidative enzymes. Therefore transcript levels of ROS-producing enzymes (lipoxygenases, NADPH oxidases), antioxidative defence enzymes (superoxide dismutases, ascorbate peroxidase, catalases) and signalling components (MAPkinases, transcription factors) were determined in leaves and roots of *Arabidopsis* wild type and *oxi1* seedlings after exposure to Cu. An overview of all genes measured and their responses to Cu is provided in Supplemental Tables 5.1. (leaves) and 5.2. (roots). In the text below, only differences caused by the mutation of *OXI1* are discussed (Figures 5.4. and 5.5.).

In leaves of *Arabidopsis* seedlings, different responses in gene expression between wild type and *oxi1* plants were observed for two antioxidative enzymes. When gene expressions for both genotypes are observed under non-exposed conditions, it can be seen that the expression of the Fe superoxide dismutase 1 gene (*FSD1*) differs between wild type and *oxi1* knockout plants under the influence of light (significant genotype difference after 4 and 6h) (Figure 5.4.). Nevertheless, in *oxi1* mutant plants exposed to Cu, transcript levels of *FSD1* were already significantly reduced at 4 and 6h after the start of exposure, whereas in wild type plants these transcript levels were only significantly decreased after 24h exposure to Cu (Figure 5.4.). Gene expression of the antioxidative enzyme catalase 1 (*CAT1*) also showed differences between non-exposed wild types and *oxi1* mutants, with increased values in the *oxi1* mutant plants during the day period (Figure 5.4.). Consequently, *CAT1* expression was downregulated in *oxi1* seedlings after 4 and 6h exposure to Cu while no changes were detected in wild type plants. Transcript levels of the pro-oxidative enzyme lipoxygenase 2 (*LOX2*) were differently affected by Cu exposure in wild type as

compared to *oxi1* mutant plants (Figure 5.4.). While the *LOX2* expression of wild type plants is significantly upregulated from 2h Cu exposure on, the *LOX2* transcript levels of *oxi1* seedlings increased only after 24h of exposure. Transcript levels of signalling components were also different between genotypes. Gene expression of *MPK3* was significantly induced in *oxi1* mutant plants from 2h Cu exposure on (Figure 5.4.). In contrast, *MPK3* expression of Cu-exposed wild type plants did not increase significantly before 24h of exposure. On the other hand, gene expression of the zinc-finger transcription factor *ZAT12* was already enhanced after 2h Cu exposure in wild type plants (Figure 5.4.), whereas in *oxi1* plants, no changes in *ZAT12* expression were observed. Finally, *oxi1* seedlings showed under control conditions remarkably reduced expression levels of the jasmonic acid inducible gene *PDF1.2* as compared to wild type plants (Figure 5.4.). However after 24h, wild type as well as *oxi1* mutant plants responded to Cu exposure with an increased *PDF1.2* expression but *PDF1.2* levels of *oxi1* mutants did not reach the levels observed in wild type plants.

In roots, expression of *FSD1* is significantly reduced in both wild type and *oxi1* plants after 24h Cu exposure (Figure 5.5.). In contrast to wild type plants, *FSD1* transcript levels of Cu exposed *oxi1* plants were already significantly decreased after 6h by 50%. A transient but significant decrease in gene expression was observed for the catalase *CAT3* in *oxi1* seedlings after 4 and 6h exposure to Cu, together with a stimulated diurnal increase in its transcript levels under control conditions (Figure 5.5.). Concerning the signalling components, genotype differences were observed for *ANP1* (Figure 5.5.). Transcript levels of the protein kinase *ANP1* were significantly elevated in wild type plants after 24h Cu exposure, while *ANP1* expression of *oxi1* plants remained at control levels. As shown in the leaves, expression levels of *PDF1.2* are lower in *oxi1* seedlings under control conditions as compared to wild type plants (Figure 5.5.). However, after 2h of Cu exposure, *PDF1.2* levels increased tremendously in *oxi1* plants while levels of wild type plants did not change. After 4h, transcript levels of *PDF1.2* increased also in Cu-exposed wild type plants. After 24h Cu exposure, *PDF1.2* expression was still elevated in wild type as well as *oxi1* plants but *PDF1.2* levels in wild type plants were significantly higher than in *oxi1* mutant plants.

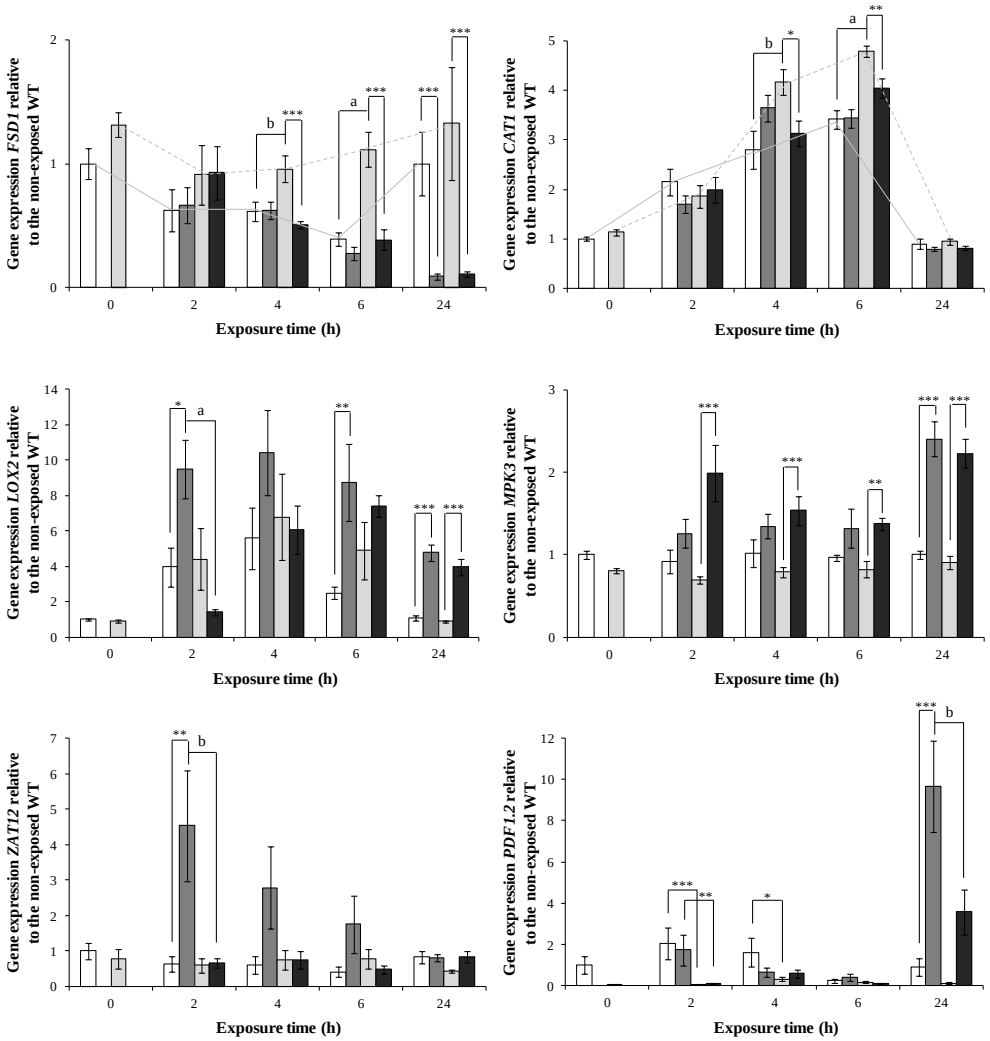

Figure 5.4.: *Transcript levels of FSD1, CAT1, LOX2, MPK3, ZAT12 and PDF1.2 in leaves of 3-week-old Arabidopsis thaliana wild type (WT) and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 2, 4, 6 and 24h (□ = wild type non-exposed, ■ = wild type exposed, ▢ = oxi1 non-exposed, ■ = oxi1 exposed). Gene expression was calculated relative to the non-exposed wildtype plants (= 1). Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: treatment effect: *: $p < 0.1$; **: $p < 0.05$; ***: $p < 0.01$; genotype and interaction effect: b: $p < 0.05$; a: $p < 0.01$). Grey line and grey dashed line represent the circadian rhythm in respectively wild type and oxi1 mutant plants.*

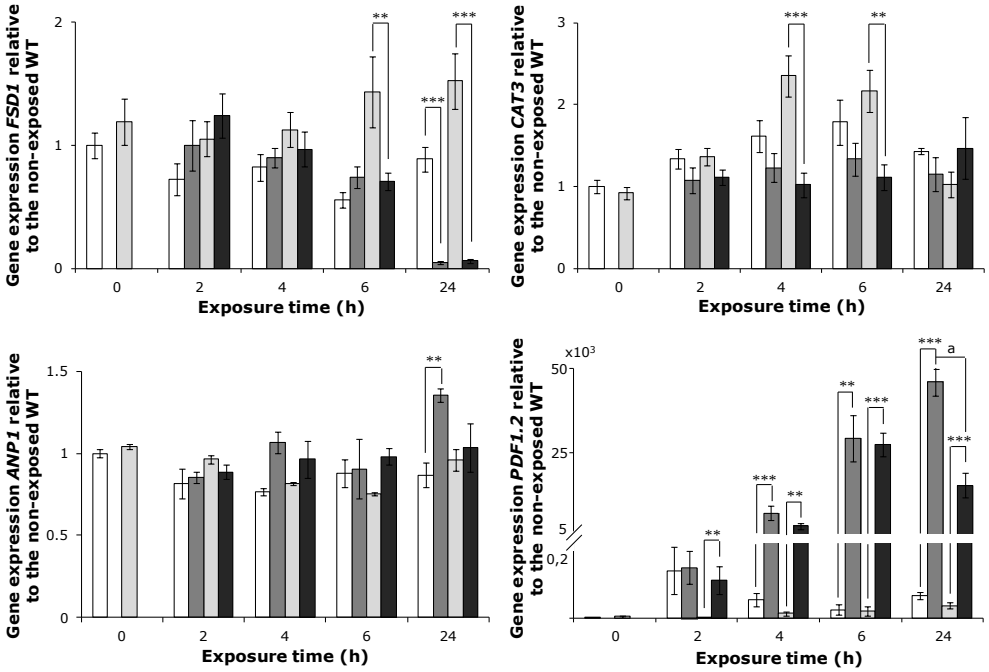

Figure 5.5.: *Transcript levels of FSD1, CAT3, ANP1 and PDF1.2 in roots of 3-week-old Arabidopsis thaliana wild type (WT) and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 2, 4, 6 and 24h (□ = wild type non-exposed, ■ = wild type exposed, □ = oxi1 non-exposed, ■ = oxi1 exposed). Gene expression was calculated relative to the non-exposed wild type plants (= 1). Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: treatment effect: **: p < 0.05; ***: p < 0.01; genotype effect: a: p < 0.01).*

5.2.5. OXI1 is involved in Cu sensitivity

To investigate if the OXI1-induced Cu responses can influence the Cu sensitivity after long-term Cu exposure, *Arabidopsis thaliana* seedlings were grown on vertical agar plates for 1 week and subsequently exposed to different Cu concentrations during 1 week. No significant difference in primary root growth was found between non-exposed wild type and *oxi1* plants (5.16 ± 0.19 vs 4.45 ± 0.19 cm). Primary root growth of wild type as well as *oxi1* mutant plants decreased gradually with increasing Cu concentration but no difference between the two genotypes was observed up to 45 µM Cu (Figure 5.6.). After 1 week exposure to 45 µM Cu, primary root growth of wild type plants was reduced with almost 70%, in contrast to *oxi1* mutant plants which showed only a 25% reduction in primary root growth. A similar difference was seen after exposure to

50 µM Cu. After exposure to 60 µM Cu, primary root growth was completely inhibited in both genotypes.

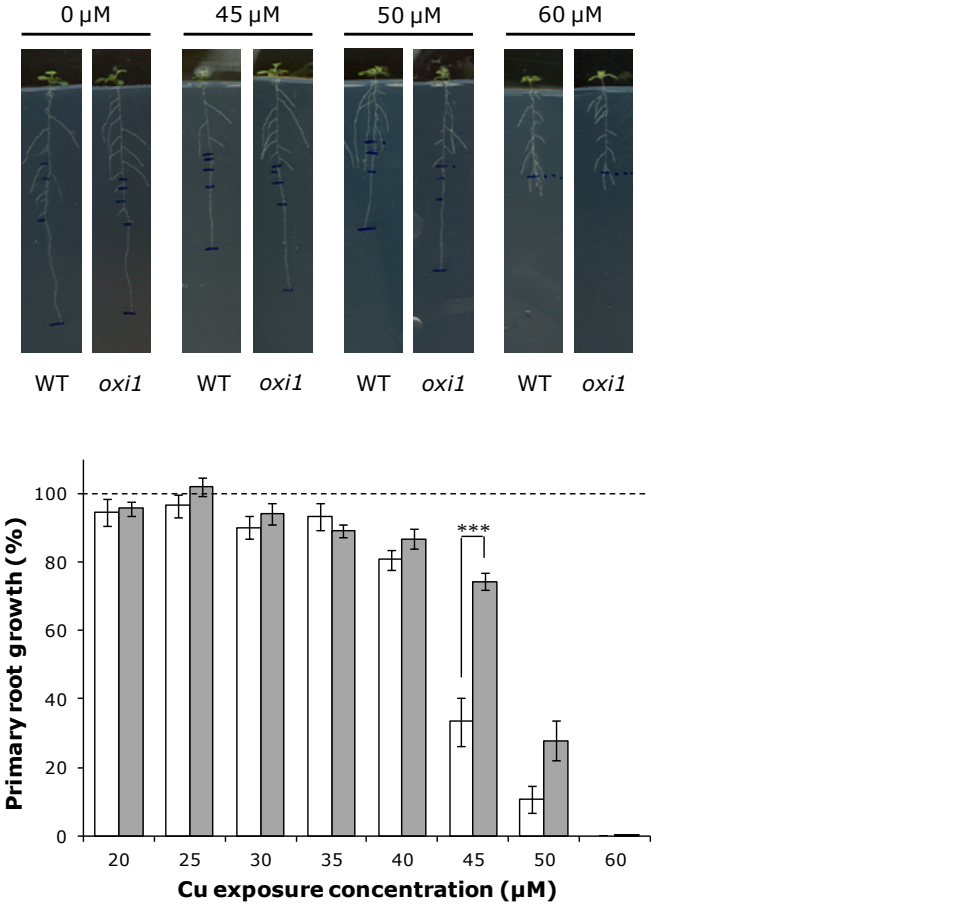

Figure 5.6.: *Root growth (%) of 2-week old Arabidopsis thaliana wild type (white bars) and oxi1 mutant (grey bars) seedlings grown on vertical agar plates and exposed to different concentrations $CuSO_4$ during 1 week. Root growth was calculated relative to the non-exposed plants (= 100%). Values are mean ± S.E. of ± 25 biologically independent replicates (significance level: ***: $p < 0.01$).*

5.3. Discussion

Earlier research (Opdenakker et al. 2012a; chapter 4) pointed out a role for OXI1 in the fast induction of Cu-mediated oxidative signalling. Therefore in this study, the function of OXI1 in Cu-induced oxidative stress responses was examined in more detail using *oxi1* knockout mutants of *Arabidopsis thaliana* (Columbia ecotype).

Exposure to Cu induced different phenotypic changes in wild type and *oxi1* knockout *Arabidopsis* seedlings. In contrast to *oxi1* mutants, wild type plants showed a significant reduction in rosette fresh weight after 24h Cu exposure whereas the Cu content of Cu-exposed *oxi1* leaves was significantly increased in contrast to wild type seedlings (Figure 5.1. and 5.2.A). In roots, the greater inhibition of root fresh weight in Cu-exposed wild type seedlings as compared to *oxi1* knockouts corresponded with the higher amount of Cu in their roots (Figure 5.1. and 5.2.B). Rentel et al. (2004) reported that *OXI1* is expressed in high levels in root hair cells of *Arabidopsis* (ecotype Columbia) plants, indicating that OXI1 is involved in normal root hair development. Since root hairs play an important role in the uptake of nutrients by increasing the root surface area (Datta et al. 2011), it can be hypothesized that OXI1 is involved in the regulation of Cu homeostasis ("restricted" uptake and translocation to above ground parts and/or exclusion) in the roots.

Since *oxi1* mutant seedlings showed alterations in leaf and root Cu content as compared to the wild types, the role of OXI1 in Cu chelation as well as in the induction of oxidative stress was investigated in leaves and roots of wild type and *oxi1* mutant plants exposed to Cu.

No clear role for OXI1 in Cu chelation could be identified

Once Cu is taken up by the root cells, the first line of defence against Cu toxicity is binding of free Cu ions to metal chelators and chaperones. This prevents the damage of biomolecules by the redox-active properties of Cu. Chelators sequester free metal ions in the cytosol or in subcellular compartments, and in this way contribute to metal detoxification (Clemens 2001, Haydon and Cobbett 2007). Metal chaperones bind and deliver metal ions to organelles and metal-requiring proteins (Grotz and Guerinot 2006, Puig et al. 2007). Murphy and Taiz (1995) tested the Cu tolerance of 10 *Arabidopsis* ecotypes and showed that Cu tolerance was closely correlated with the expression level of a 2-type metallothionein (MT2) gene. Other authors reported that MT gene expression is strongly induced upon Cu treatment (Zhou and Goldsbrough 1994, Hsieh et al. 1995, Roosens et al. 2004, Guo et al. 2008). In contrast, the role of PCs in Cu detoxification is not clearly identified. Cu was reported to be a strong activator of PC biosynthesis and Cu was already found to form complexes with PCs

(Cobbett and Goldsbrough 2002). Therefore, gene expression of MTs and PC synthases was measured in wild type and *oxi1* mutant plants after exposure to Cu.

Only in the leaves, differences between wild type and *oxi1* mutant plants were seen in the gene expression of metallothioneins, more specific *MT1a* and *MT1c*. Both genes were downregulated faster in *oxi1* mutant plants than in wild type plants (Figure 5.2.C and D). Thus, OXI1 can be responsible for the control of steady state mRNA levels of MT1 in leaves of *Arabidopsis*, but this downregulation cannot explain the higher Cu accumulation in the leaves of *oxi1* mutant plants.

Interplay between OXI1 and Cu at the cellular level: damage versus signalling

At the cellular level, elevated concentrations of Cu are known to induce oxidative stress in plants (Smeets et al. 2005, Cuypers et al. 2009). OXI1 is a signalling molecule, able to induce MAPK cascades in ROS-mediated processes (Rentel et al. 2004, Anthony et al. 2006, Petersen et al. 2009, Forzani et al. 2011). Therefore, it was examined whether OXI1 is involved in oxidative stress signalling and regulation of the cellular redox state of *Arabidopsis* plants exposed to Cu.

First, it was investigated whether OXI1 controls the gene expression of downstream signalling targets, which we identified in previous work as being regulated in the same way as *OXI1* upon Cu exposure (Opdenakker et al. 2012a; chapter 4). Concerning the downstream kinases MPK3 and 6, which are also defined as targets of OXI1 (Rentel et al. 2004), we found only evidence for a regulation of *MPK3* by OXI1 under Cu stress. In leaves of *oxi1* knockout plants, *MPK3* gene expression was induced earlier after Cu exposure as compared to wild type seedlings (Figure 5.4.), suggesting that a bypass via other signalling components can take place. With regard to the transcription factors, only *ZAT12* transcript levels in the leaves were affected by the *oxi1* mutation (Figure 5.4.), suggesting that OXI1 is responsible for the immediate induction of *ZAT12* expression after Cu exposure. In the roots, gene expression of the protein kinase *ANP1* was affected in *oxi1* mutant plants after Cu exposure (Figure 5.5.). ANP1 is, like OXI1, a MAP3K able to induce a phosphorylation cascade involving

MPK3 and MPK6 (Kovtun et al. 2000). In wild type plants, *ANP1* transcript levels are increased after 24h exposure to Cu while this induction was aborted in *oxi1* mutant plants (Figure 5.5.) suggesting that regulation of *ANP1* expression after Cu exposure is under the control of OXI1.

Secondly, the effect of the OXI1 mutation on the cellular redox state was investigated. We previously reported that even though Cu was not translocated to the leaves in wild type plants, changes in gene expression of pro- and antioxidative genes in the leaves were evident of root-to-shoot signalling (Remans et al. 2010, Cuypers et al. 2011). However, here we show that the involvement of OXI1 in this root-to-shoot signalling cannot easily be revealed: since the *OXI1* mutation causes a higher translocation of Cu to the leaves, it cannot be distinguished whether changes in gene expression were due to a direct effect of the *OXI1* mutation, or due to a secondary effect of higher Cu contents in the leaves.

Transcript levels of antioxidative enzymes in leaves (*FSD1, CAT1*) and roots (*FSD1, CAT3*) of *oxi1* seedlings were different as compared to wild types under Cu stress (Figures 5.4. and 5.5.). A downregulation of the transcript levels was seen earlier in *oxi1* mutants than in wild type plants. In leaves, this was due to a higher *FSD1* and *CAT1* expression in non-exposed *oxi1* plants as compared to non-exposed wild types (Figure 5.4.). This suggests that OXI1 plays a role in light-regulated transcription of *FSD1* and *CAT1* under non-exposed conditions but it is not involved in transcriptional control of these genes after Cu exposure. In the roots, a similar effect was noticed for the gene expression levels of *FSD1* and *CAT3* (Figure 5.5.). According to these findings, light-driven regulation of the antioxidative system of the cell is clearly regulated by a.o. OXI1.

A decrease in the levels of antioxidants can disturb the cellular redox state thereby inducing oxidative stress, which can result in oxidative signalling on one hand or oxidative damage to cellular components on the other hand. As a parameter of oxidative damage, the amount of lipid peroxidation was determined. In leaves of both wild type and *oxi1* plants, no differences in lipid peroxidation were observed. In contrast, roots of *oxi1* mutants showed lower levels of TBArm (Figure 5.3.), indicative of a lower level of membrane damage, than wild type plants. This can be linked to a higher Cu amount in wild type roots, which in turn leads to higher ROS production and hence membrane

damage in wild type plants. In leaves however, membrane integrity is maintained regardless of a higher Cu content in *oxi1* mutants.

Lipid peroxidation can be mediated by Cu-induced ROS production but can also be initiated by lipoxygenases that also take part in lipid signalling. These are enzymes that catalyze the oxygenation of polyunsaturated fatty acids into lipid hydroperoxides, which are involved in responses to different stresses (Skorzynska-Polit et al. 2006). These lipid hydroperoxides can be further metabolized by different pathways and the resulting oxygenated derivatives are collectively called oxylipins (Andreou et al. 2009). In our study, gene expression of *LOX2* was determined in leaves as it was the most abundantly expressed isoform (Remans et al. 2010). Furthermore, it initiates jasmonate (JA) biosynthesis, which is also known to be regulated by MAPKinase cascades (Opdenakker et al. 2012b; chapter 1). In leaves of wild type plants, OXI1 is involved in the early upregulation of *LOX2* gene expression (Figure 5.4.), which suggests its involvement in altered oxylipin signalling after exposure of plants to excess Cu (Mithöfer et al. 2004, Montillet et al. 2004, Remans et al. 2010).

In particular JA, one of the oxylipins produced after lipoxygenase action, is known to be involved in the response of *Arabidopsis* to toxic metals, including Cu (Xiang and Oliver 1998, Maksymiec and Krupa 2006, Maksymiec et al. 2007), and LOX2 has been attributed 13-LOX activity at the origin of JA biosynthesis upon wounding (Bell et al. 1995). One of the best known targets of JA signalling is the plant defensin PDF1.2, whose mRNA accumulation can be positively or negatively regulated by JA (Wasternack 2007, Zarei et al. 2011). In our study, mRNA levels of *PDF1*.2 were strongly reduced in leaves of *oxi1* mutant plants under non-exposed conditions (Figure 5.4.) indicating that OXI1 plays a role in the transcriptional control of *PDF1*.2. Even after 24h Cu exposure, *PDF1*.2 transcript levels were lower in *oxi1* mutant seedlings as compared to wild type plants (Figure 5.4.) showing that OXI1 is also important in the regulation of *PDF1*.2 after Cu exposure. From these data we can conclude that in *Arabidopsis* leaves OXI1 is at least partially responsible for the induction of oxylipin signalling via regulation of *LOX2*.

From these data, it is evident that OXI1 is important in the regulation of Cu stress responses in *Arabidopsis thaliana* after short-term Cu exposure. Whether

these OXI1-mediated plant responses to Cu can lead to a better Cu acclimation and/or tolerance after long-term exposure was investigated using root growth assays on vertical agar plates. This experimental set-up was earlier used by Schiavon et al. (2007) to analyze variations in Cu sensitivity between different *Arabidopsis thaliana* accessions (Columbia, Landsberg *erecta*, Wassilewskija). Using this primary root growth inhibition in vertical agar plates, we report that *oxi1* mutants are less sensitive to excess Cu exposure than wild type plants (Figure 5.6.).

Based on our findings, we suggest that alterations in OXI1-mediated Cu uptake and translocation on one hand and signal transduction on the other hand, disturb specific plant responses and hence plant sensitivity to Cu.

Supplemental Table 5.1.: *Transcript levels in leaves of 3-week-old Arabidopsis thaliana wild type and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 2, 4, 6 and 24h. Gene expression was calculated relative to the non-exposed wild type plants (= 1). Values are mean ± S.E. of ± 5 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulation:* p<0.01 | p < 0.001 *; upregulation:* p<0.01 | p < 0.001 *).*

LEAVES										
	0h		2h				4h			
			wild type		*oxi1*		wild type		*oxi1*	
Gene	wild type	*oxi1*	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
LOX2	1.00±0.06	0.92±0.10	3.96±1.11	9.48±1.64	4.41±1.73	1.41±0.18	5.58±1.74	10.4±2.41	6.77±2.44	6.07±1.34
RBOHC	1.00±0.25	0.38±0.06	2.01±0.71	5.61±1.95	0.46±0.14	0.28±0.09	3.36±1.86	5.22±1.59	0.33±0.11	1.85±0.94
RBOHD	1.00±0.07	0.95±0.10	1.00±0.06	1.89±0.12	0.88±0.11	1.29±0.07	0.88±0.18	1.50±0.23	0.88±0.11	1.38±0.20
CSD1	1.00±0.06	0.84±0.03	1.21±0.14	1.12±0.18	0.84±0.08	0.91±0.06	1.12±0.17	1.14±0.19	1.09±0.05	0.95±0.07
CSD2	1.00±0.09	0.74±0.07	0.83±0.08	0.93±0.16	0.52±0.07	0.72±0.09	0.53±0.07	0.62±0.07	0.73±0.06	0.46±0.05
FSD1	1.00±0.13	1.31±0.10	0.63±0.17	0.67±0.15	0.91±0.24	0.93±0.21	0.62±0.08	0.63±0.07	0.96±0.10	0.51±0.03
CAT1	1.00±0.04	1.14±0.06	2.15±0.27	1.70±0.18	1.86±0.23	1.99±0.26	2.80±0.38	3.64±0.26	4.17±0.26	3.13±0.25
CAT2	1.00±0.06	0.99±0.05	0.49±0.04	0.38±0.03	0.43±0.03	0.37±0.03	0.15±0.01	0.14±0.01	0.20±0.01	0.13±0.01
CAT3	1.00±0.05	0.79±0.06	4.20±0.55	4.04±0.31	3.73±0.58	3.90±0.46	6.38±0.68	8.29±0.23	8.93±1.05	9.79±1.03
APX1	1.00±0.06	1.06±0.07	1.41±0.07	1.44±0.15	1.37±0.19	1.10±0.16	0.75±0.07	0.86±0.10	1.22±0.14	0.91±0.09
ANP1	1.00±0.06	1.13±0.09	1.12±0.09	1.08±0.09	1.00±0.07	1.09±0.05	0.92±0.08	0.96±0.05	1.11±0.05	0.90±0.02
MPK3	1.00±0.05	0.80±0.03	0.92±0.15	1.26±0.17	0.69±0.04	1.99±0.34	1.02±0.17	1.35±0.15	0.79±0.06	1.54±0.18
MPK6	1.00±0.02	1.01±0.02	0.98±0.03	1.01±0.06	0.90±0.06	1.00±0.04	0.96±0.06	1.11±0.07	1.05±0.06	1.00±0.02
WRKY22	1.00±0.05	0.97±0.05	0.89±0.07	1.68±0.28	0.68±0.07	1.79±0.27	0.76±0.09	0.67±0.11	0.69±0.04	0.48±0.10
WRKY25	1.00±0.08	0.84±0.05	1.11±0.16	3.02±0.56	1.06±0.18	3.04±0.25	1.81±0.17	4.25±0.57	1.40±0.19	3.06±0.34
ZAT12	1.00±0.23	0.78±0.27	0.63±0.22	4.54±1.56	0.60±0.21	0.67±0.13	0.60±0.25	2.79±1.17	0.76±0.27	0.75±0.25
PDF1.2	1.00±0.43	0.08±0.02	2.05±0.78	1.73±0.74	0.07±0.01	0.11±0.02	1.62±0.69	0.66±0.23	0.33±0.11	0.59±0.19
PCS1	1.00±0.06	1.02±0.08	1.20±0.11	1.78±0.45	1.05±0.07	2.71±0.09	1.28±0.20	1.40±0.15	1.16±0.03	1.52±0.10
MT1a	1.00±0.13	1.12±0.06	0.65±0.20	1.07±0.08	1.49±0.08	0.94±0.04	1.14±0.08	1.03±0.11	1.31±0.05	1.09±0.02
MT1c	1.00±0.08	1.10±0.05	1.21±0.09	1.04±0.09	1.23±0.05	0.76±0.05	0.69±0.07	0.59±0.09	0.68±0.03	0.39±0.04
MT2a	1.00±0.03	1.35±0.13	0.92±0.09	0.76±0.04	0.92±0.06	0.92±0.08	0.83±0.11	0.96±0.09	0.86±0.10	1.01±0.08
MT2b	1.00±0.03	1.14±0.05	1.30±0.08	1.02±0.04	1.15±0.06	1.08±0.09	0.95±0.11	0.84±0.04	1.04±0.06	0.86±0.04
MT3	1.00±0.02	1.04±0.03	0.89±0.09	0.86±0.03	0.99±0.05	0.98±0.10	0.85±0.08	0.84±0.10	0.98±0.05	0.90±0.04

Supplemental Table 5.1.: *Continued.*

LEAVES								
	6h				24h			
	wild type		*oxi1*		wild type		*oxi1*	
Gene	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
LOX2	2.50±0.33	8.74±2.16	4.90±1.62	7.43±0.61	1.07±0.14	4.77±0.46	0.89±0.07	3.96±0.48
RBOHC	1.04±0.42	9.25±4.70	0.40±0.14	0.80±0.51	1.26±0.59	1.28±0.73	0.17±0.03	0.42±0.18
RBOHD	0.66±0.03	1.73±0.24	0.66±0.06	1.18±0.05	1.36±0.03	2.50±0.21	1.09±0.03	2.06±0.26
CSD1	1.19±0.08	1.04±0.08	0.79±0.08	0.83±0.06	1.14±0.06	1.55±0.13	1.00±0.08	1.69±0.04
CSD2	0.66±0.12	0.38±0.04	0.52±0.05	0.42±0.04	0.99±0.11	0.58±0.03	0.84±0.09	0.57±0.03
FSD1	0.39±0.05	0.28±0.06	1.12±0.14	0.39±0.09	1.00±0.26	0.09±0.02	1.33±0.46	0.11±0.02
CAT1	3.41±0.19	3.44±0.19	4.79±0.12	4.04±0.19	0.90±0.10	0.80±0.03	0.95±0.06	0.82±0.04
CAT2	0.09±0.01	0.09±0.01	0.08±0.01	0.06±0.01	1.04±0.08	0.58±0.06	1.03±0.08	0.66±0.04
CAT3	9.51±0.23	13.8±2.57	9.12±1.28	7.54±1.14	1.00±0.10	3.13±0.32	0.85±0.16	2.32±0.36
APX1	0.84±0.10	0.90±0.03	1.08±0.03	1.13±0.08	1.44±0.18	1.37±0.13	1.29±0.16	1.56±0.15
ANP1	1.13±0.09	1.18±0.14	1.10±0.08	1.18±0.03	1.21±0.11	0.85±0.02	1.09±0.10	0.88±0.08
MPK3	0.97±0.04	1.32±0.23	0.82±0.10	1.37±0.07	1.00±0.05	2.40±0.21	0.90±0.08	2.23±0.17
MPK6	1.03±0.03	1.02±0.03	1.00±0.03	0.94±0.02	1.00±0.06	0.87±0.03	0.97±0.11	0.96±0.04
WRKY22	0.65±0.03	0.33±0.04	0.76±0.05	0.17±0.06	1.00±0.20	3.13±0.64	0.63±0.06	3.48±0.47
WRKY25	1.91±0.20	1.20±0.42	1.28±0.07	0.30±0.08	0.82±0.12	1.85±0.25	0.61±0.06	2.93±0.62
ZAT12	0.41±0.13	1.76±0.81	0.77±0.27	0.48±0.11	0.83±0.18	0.80±0.11	0.43±0.05	0.85±0.16
PDF1.2	0.25±0.11	0.40±0.16	0.19±0.05	0.13±0.03	0.91±0.41	9.66±2.23	0.13±0.04	3.58±1.09
PCS1	1.48±0.04	2.16±0.22	1.78±0.20	1.82±0.10	1.09±0.07	1.35±0.10	1.17±0.07	1.34±0.08
MT1a	1.95±0.12	1.41±0.24	2.37±0.17	1.59±0.17	0.93±0.07	0.65±0.04	0.83±0.04	0.92±0.04
MT1c	0.39±0.05	0.39±0.07	0.45±0.01	0.25±0.03	1.11±0.09	0.20±0.04	1.18±0.11	0.31±0.04
MT2a	1.55±0.23	2.80±0.52	2.38±0.12	3.38±0.29	1.67±0.07	3.89±0.18	1.60±0.20	3.68±0.18
MT2b	0.81±0.04	0.95±0.03	0.92±0.08	0.90±0.01	1.33±0.17	1.27±0.05	1.18±0.14	1.44±0.13
MT3	0.90±0.04	1.04±0.07	0.96±0.07	1.00±0.03	1.09±0.08	1.74±0.11	1.14±0.06	1.74±0.14

Supplemental Table 5.2.: *Transcript levels in roots of 3-week-old Arabidopsis thaliana wild type and oxi1 mutant plants exposed to 2 µM $CuSO_4$ during 2, 4, 6 and 24h. Gene expression was calculated relative to the non-exposed wild type plants (= 1). Values are mean ± S.E. of ± 5 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulation:* p<0.01 | p < 0.001 *; upregulation:* p<0.01 | p < 0.001 *).*

ROOTS										
	0h		2h				4h			
			wild type		*oxi1*		wild type		*oxi1*	
Gene	wild type	*oxi1*	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
LOX1	1.00±0.06	1.11±0.07	1.12±0.10	6.84±0.76	1.02±0.05	6.31±0.72	1.01±0.12	65.5±6.09	0.96±0.04	57.7±6.54
RBOHC	1.00±0.08	1.28±0.02	0.68±0.03	1.26±0.09	1.02±0.07	1.57±0.09	0.72±0.09	2.19±0.18	0.87±0.03	2.26±0.20
RBOHD	1.00±0.07	0.92±0.05	0.77±0.23	13.1±0.91	0.89±0.15	11.1±0.90	0.66±0.06	12.5±2.37	0.64±0.04	9.49±2.48
CSD1	1.00±0.03	1.06±0.03	1.03±0.03	1.40±0.05	1.17±0.04	1.47±0.01	1.10±0.07	1.64±0.13	0.95±0.05	1.84±0.05
CSD2	1.00±0.02	0.97±0.03	0.93±0.02	0.97±0.02	0.94±0.04	0.98±0.03	0.84±0.02	0.93±0.04	0.75±0.02	0.92±0.03
FSD1	1.00±0.11	1.19±0.19	0.72±0.13	1.00±0.20	1.05±0.14	1.25±0.18	0.83±0.11	0.90±0.08	1.13±0.14	0.97±0.14
CAT1	1.00±0.05	0.90±0.04	0.77±0.16	1.61±0.30	0.84±0.04	1.45±0.08	1.41±0.14	4.02±0.74	1.49±0.11	4.06±0.61
CAT2	1.00±0.08	1.02±0.05	0.92±0.06	0.43±0.04	1.10±0.05	0.54±0.03	0.84±0.08	0.37±0.04	1.00±0.02	0.37±0.03
CAT3	1.00±0.09	0.92±0.07	1.34±0.12	1.07±0.16	1.36±0.11	1.11±0.10	1.62±0.19	1.23±0.17	2.35±0.25	1.02±0.15
APX1	1.00±0.03	0.89±0.04	0.93±0.02	1.36±0.02	1.01±0.05	1.59±0.06	0.83±0.09	1.46±0.12	0.81±0.01	1.50±0.05
ANP1	1.00±0.02	1.04±0.01	0.82±0.09	0.85±0.03	0.96±0.02	0.88±0.04	0.76±0.02	1.06±0.07	0.81±0.01	0.96±0.11
MPK3	1.00±0.06	0.95±0.04	0.88±0.07	10.0±1.06	0.88±0.05	10.8±0.70	1.00±0.03	5.80±0.37	0.86±0.04	5.39±0.44
MPK6	1.00±0.03	0.95±0.02	0.94±0.03	1.15±0.04	1.00±0.01	1.20±0.03	0.93±0.02	1.52±0.04	0.95±0.03	1.48±0.07
WRKY22	1.00±0.07	1.06±0.04	0.90±0.09	3.66±0.23	1.08±0.11	3.55±0.22	0.90±0.04	3.80±0.31	1.04±0.10	3.66±0.12
WRKY25	1.00±0.05	1.23±0.06	1.07±0.08	6.94±0.38	1.36±0.06	7.90±0.42	1.39±0.04	10.2±0.42	1.40±0.09	10.6±0.52
ZAT12	1.00±0.26	0.67±0.10	0.29±0.03	204±33.6	0.48±0.03	189±12.2	0.27±0.06	419±37.7	0.25±0.04	545±83.7
PDF1.2	1.00±0.66	4.12±2.53	127±64.4	135±44.0	0.10±0.09	101±36.2	47.9±17.5	7188±2116	11.6±5.36	3296±912
PCS1	1.00±0.03	1.00±0.05	0.71±0.07	4.19±0.58	0.95±0.03	6.13±0.49	0.83±0.07	3.05±0.36	0.99±0.09	3.13±0.35
MT1a	1.00±0.10	1.09±0.08	0.69±0.23	1.16±0.29	0.80±0.08	1.22±0.10	0.85±0.20	3.83±0.44	0.75±0.07	2.96±0.16
MT1c	1.00±0.12	1.20±0.14	0.81±0.08	0.61±0.11	1.06±0.05	0.94±0.06	0.88±0.09	0.51±0.08	0.99±0.07	0.53±0.02
MT2a	1.00±0.17	1.37±0.35	0.79±0.09	0.70±0.01	0.65±0.04	0.67±0.05	1.68±0.41	2.84±1.02	3.36±0.67	2.49±0.40
MT2b	1.00±0.03	1.00±0.10	1.22±0.14	1.22±0.05	1.02±0.05	1.19±0.03	0.86±0.04	1.20±0.11	0.94±0.01	1.15±0.16
MT3	1.00±0.05	0.95±0.07	1.28±0.04	1.13±0.12	1.49±0.07	1.37±0.02	1.34±0.11	0.45±0.09	1.49±0.13	0.38±0.04

Supplemental Table 5.2.: *Continued.*

ROOTS								
	6h				24h			
	wild type		*oxi1*		wild type		*oxi1*	
Gene	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
LOX1	1.34±0.18	93.5±10.9	1.24±0.15	99.5±2.74	2.24±0.18	101±10.2	1.79±0.20	111±9.73
RBOHC	0.78±0.09	1.56±0.15	1.01±0.05	1.77±0.13	0.95±0.07	0.28±0.02	1.07±0.03	0.20±0.01
RBOHD	0.69±0.02	11.0±2.33	0.66±0.13	10.7±1.41	1.00±0.07	11.3±1.11	0.75±0.09	10.2±1.40
CSD1	1.11±0.09	2.22±0.14	1.02±0.02	2.16±0.02	1.28±0.06	2.86±0.11	1.20±0.06	2.73±0.15
CSD2	0.87±0.03	0.88±0.03	0.77±0.01	0.79±0.02	1.09±0.03	1.36±0.07	0.97±0.02	1.20±0.06
FSD1	0.56±0.07	0.75±0.09	1.43±0.29	0.71±0.07	0.89±0.10	0.05±0.01	1.52±0.22	0.06±0.02
CAT1	1.21±0.13	3.89±0.50	1.56±0.10	3.68±0.67	0.30±0.03	4.60±1.06	0.27±0.02	4.14±0.41
CAT2	0.67±0.10	0.22±0.02	0.93±0.03	0.27±0.01	1.02±0.06	0.17±0.03	0.88±0.07	0.22±0.02
CAT3	1.79±0.27	1.30±0.20	2.17±0.25	1.11±0.16	1.43±0.04	1.15±0.21	1.03±0.15	1.47±0.37
APX1	0.77±0.07	1.19±0.13	0.76±0.01	1.42±0.03	0.92±0.05	1.43±0.12	0.92±0.05	1.21±0.06
ANP1	0.88±0.08	0.91±0.18	0.75±0.01	0.98±0.05	0.87±0.07	1.36±0.04	0.96±0.06	1.04±0.15
MPK3	0.88±0.04	3.92±0.45	0.83±0.05	3.90±0.14	0.83±0.04	2.72±0.31	0.69±0.03	2.48±0.12
MPK6	0.88±0.04	1.32±0.07	0.92±0.03	1.32±0.03	0.96±0.02	0.93±0.04	0.85±0.01	0.86±0.04
WRKY22	0.79±0.08	2.47±0.26	0.96±0.04	2.45±0.07	0.96±0.11	3.65±0.17	0.90±0.10	3.62±0.28
WRKY25	1.20±0.11	11.2±0.74	1.56±0.06	9.94±0.21	1.04±0.05	6.33±0.48	1.16±0.05	5.51±0.31
ZAT12	0.49±0.08	156±58.9	0.22±0.10	92.4±46.1	0.02±0.02	10.0±7.11	0.01±0.01	2.76±2.19
PDF1.2	22.5±13.8	29454±6893	18.8±12.0	27483±3592	58.6±9.88	46270±4312	33.5±9.08	15460±3787
PCS1	0.82±0.07	1.98±0.15	0.95±0.01	2.29±0.20	0.95±0.05	1.18±0.15	0.85±0.07	1.28±0.14
MT1a	0.59±0.06	5.40±1.39	0.81±0.14	5.19±0.45	0.66±0.05	51.8±7.10	0.43±0.06	63.7±7.96
MT1c	0.92±0.11	0.33±0.08	0.83±0.06	0.42±0.04	1.64±0.09	0.11±0.02	1.51±0.13	0.12±0.02
MT2a	2.10±0.39	2.14±0.26	7.48±2.85	3.81±1.30	0.97±0.35	4.30±1.68	1.64±0.61	7.08±1.86
MT2b	0.84±0.05	1.13±0.11	0.77±0.03	1.08±0.06	1.07±0.06	2.61±0.17	0.92±0.03	2.98±0.39
MT3	1.35±0.16	0.25±0.01	1.27±0.18	0.27±0.01	1.48±0.10	1.61±0.16	1.34±0.14	1.72±0.40

Chapter 6

The protein kinase MPK6 mediates Cu-specific oxidative signalling in *Arabidopsis thaliana*

Kelly Opdenakker, Tony Remans, Heribert Hirt, Jaco Vangronsveld, Ann Cuypers (2012). The protein kinase MPK6 mediates Cu-specific oxidative signalling 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 signalling molecules in the cell. ROS signalling in plants controls the transcription of target genes involved in ROS scavenging or production by the use of mitogen-activated protein kinase (MAPK) pathways. Here, we describe the role of the MAPKinase MPK6 in Cu-induced oxidative stress. Although *mpk6* knockout seedlings were observed to be more Cu tolerant than wild type plants, their roots experienced more oxidative stress after Cu exposure. It is hypothesized that these oxidative stress signals are used for root-to-shoot signalling, inducing acclimation responses to Cu exposure. In leaves of Cu-exposed *mpk6* mutants, it was demonstrated that they accumulated significantly higher amounts of Cu as compared to leaves of wild type plants, resulting in elevated H_2O_2 levels. However, no oxidative damage was detected suggesting that the higher ROS concentrations are used for oxidative signalling. In addition, MPK6 was observed to regulate the transcription of the pro-oxidative enzymes, *RBOHC* and *RBOHD*, and the antioxidative enzymes, *CSD1*, *CSD2*, *CAT1* and *CAT3*, via the WRKY transcription factors, WRKY22 and WRKY25, or SPL7- and miRNA398-mediated processes.

6.1. Introduction

Elevated concentrations of copper (Cu) are locally present in the environment as a consequence of mining and industrial processes or the agricultural use of pesticides and fungicides (Kirkham 2006, Chary *et al*. 2008, Ruttens *et al*. 2011). Although Cu is an essential trace element for the plant, it becomes toxic and induces stress effects in plants at elevated concentrations. The main visible symptoms of metal toxicity are leaf chlorosis and growth inhibition (Cuypers *et al*. 2009, Yruela 2009). At the cellular level, metals can disturb the cellular redox status, leading to an overproduction of reactive oxygen species (ROS), which induce oxidative stress (Smeets *et al*. 2009, Cuypers *et al*. 2011a). Because Cu is a redox-active metal, it has the ability to produce ROS directly via Fenton and Haber-Weiss reactions (Kehrer 2000). Although the rate of ROS production is elevated during stress conditions, low levels of ROS are essential for normal cell metabolism. This requires a tight regulation of subcellular ROS levels by the antioxidative defence system of the cell (Mittler *et al*. 2004, Cuypers *et al*. 2011b).

As signalling molecules, ROS play a key role in processes mediating defence against biotic and abiotic stresses (Torres and Dangl 2005) but also during normal cell growth and development (Foreman *et al*. 2003). Downstream signalling events associated with ROS sensing involve activation of mitogen-activated protein kinase (MAPK) pathways (Mittler *et al*. 2004, Apel and Hirt 2004, Opdenakker *et al*. 2012b: chapter 1).

Stimulation of MAPK cascades, and more specifically MPK6, by ROS is reported in different studies (Kovtun *et al*. 2000, Rentel *et al*. 2004). Activation of MPK6 is also associated with Cu stress in plants. In *Medicago sativa* roots as well as in rice roots, the orthologues of *Arabidopsis* MPK6, SIMK and OsMPK6, were activated after exposure to excess Cu ions (Jonak *et al*. 2004, Yeh *et al*. 2007). Opdenakker *et al*. (2012a; chapter 4) showed that 24h exposure of *Arabidopsis thaliana* seedlings to environmental realistic concentrations of Cu (2 µM) induced transcript levels of *MPK6* in roots of *Arabidopsis* plants.

The function of MAPKinases, like MPK6, is to regulate the activity of transcription factors, leading to transcriptional control of target genes. For example, MPK6 activated by a flagellin induced MAPK cascade (MEKK1-MKK4/MKK5-MPK6) is known to activate the WRKY transcription factors, WRKY22 and WRKY29 (Asai *et*

al. 2002). Furthermore, short-term exposure of three-week-old *Arabidopsis* plants to 2 µM Cu induced gene expression of *WRKY22*, *WRKY25*, *WRKY29* and *ZAT12* in leaves and roots. These data suggest that these transcription factors play an essential role in regulation of the stress response upon metal exposure (Opdenakker *et al*. 2012a, chapter 4). Several enzymes, involved in controlling the redox status of the cell, are reported to be regulated at the transcriptional level by WRKY25 and ZAT12. Examples are the ascorbate peroxidases *APX1* and *APX2,* the superoxide dismutases *CSD1*, *CSD2* and *FSD1*, and the pro-oxidative NADPH oxidase *RBOHD* and lipoxygenase *LOX4* (Rhizhsky *et al*. 2004, Davletova *et al*. 2005, Vogel *et al*. 2005, Li *et al*. 2009).

From the above-mentioned literature, it is known that Cu stress induces gene expression of *MPK6* and enhances its activity in plants. On the other hand it has been shown that (1) exposure of plants to increased concentrations of Cu induces ROS production, eventually leading to oxidative stress in the plant cells, and (2) MPK6 is activated by ROS and can regulate gene expression of pro- and antioxidative enzymes via activation of WRKY transcription factors. Based on these findings, we hypothesize that MPK6 is also activated by the Cu-mediated ROS production in *Arabidopsis* and that it plays a central role in the regulation of the cellular redox status after Cu exposure. Therefore, we exposed *Arabidopsis* wild type as well as *mpk6* knockout plants to a sublethal Cu concentration and investigated the effect of the loss of MPK6 on the cellular redox status at different biological organisation levels.

6.2. Results

6.2.1. mpk6 knockout mutants are more tolerant to Cu stress

In an initial experiment, the Cu sensitivity of *mpk6* knockout plants was tested by analysing root growth. Therefore, *Arabidopsis thaliana* seedlings, grown on vertical agar plates for 1 week, were exposed to different Cu concentrations during 1 week (Figure 6.1.). Primary root growth showed no significant difference between non-exposed wild type and *mpk6* mutant plants (8.29 ± 0.20 vs 9.24 ± 0.25 cm). After exposure to Cu, primary root growth of wild type and *mpk6* seedlings decreased gradually with increasing Cu concentration ($p < 0.0001$ for the decrease in root growth after exposure to each Cu

concentration). However, primary root growth of *mpk6* mutant plants was less affected by Cu exposure. After exposure to 2, 5 or 10 µM Cu during 1 week, primary root length of wild type plants was reduced by almost 50%. In contrast, *mpk6* mutants showed a reduction of only 20%, indicating a significant higher primary root growth than wild type plants after exposure to 2, 5 or 10 µM Cu. Exposure to 15 µM Cu inhibited the primary root growth strongly in both genotypes but still root growth of *mpk6* seedlings was significantly higher than in wild type plants. After exposure to 20 µM Cu, primary root growth was completely abolished in wild type as well as in *mpk6* mutant plants.

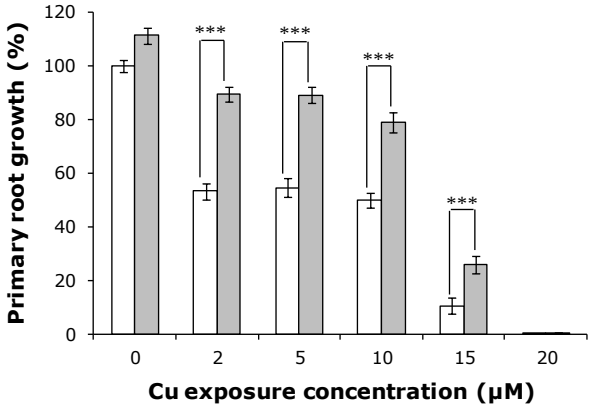

Figure 6.1.: *Root growth (%) of 2-week old Arabidopsis thaliana wild type (white bars) and mpk6 knockout (grey bars) seedlings grown on vertical agar plates and exposed to different concentrations $CuSO_4$ during 1 week. Root growth was calculated relative to the non-exposed wild type plants (= 100%). Values are mean ± S.E. of ± 25 biologically independent replicates (significance level: ***: $p < 0.01$).*

Three-week-old *Arabidopsis* seedlings, grown in hydroponics and exposed to 2 µM Cu during 2 or 24h, showed a decreasing trend in leaf and root weight in both wild type and *mpk6* knockout plants after 24h (data not shown). However, no differences between the two genotypes were observed.

6.2.2. MPK6 affects the Cu content of leaves

Because wild type *Arabidopsis* plants were more sensitive to Cu exposure than *mpk6* knockout plants, the concentration of Cu as well as of other essential elements was determined in roots and leaves of 3-week-old hydroponically grown *Arabidopsis* seedlings exposed to 2 µM Cu during 24h (Table 6.1.). In roots, no differences were observed in Cu content between non-exposed wild type and *mpk6* mutant plants. After 24h exposure to Cu, both genotypes showed similarly elevated Cu concentrations in their roots. In leaves, *mpk6*

mutants showed, in contrast to wild types, a significantly increased Cu concentration in the leaves after exposure to Cu.

Table 6.1.: *Element concentrations (mg kgDW^{-1}) in leaves and roots of 3-week-old Arabidopsis wild type and mpk6 knockout seedlings grown on hydroponics and exposed to 2 µM $CuSO_4$ during 24h. Values are mean ± S.E. of at least 5 biologically independent replicates (significance levels: ***: p < 0.01, **: p < 0.05).*

ROOTS				
	wild type		*mpk6*	
Element	Control	2 µM Cu	Control	2 µM Cu
Cu	27 ± 2	1220 ± 79***	22 ± 1	1418 ± 110***
Zn	442 ± 52	395 ± 65	336 ± 33	277 ± 14
K	60662 ± 1372	37266 ± 5650**	63568 ± 1947	36495 ± 7889**
Ca	2203 ± 210	5241 ± 1109***	2137 ± 218	4134 ± 696**
Fe	695 ± 89	1136 ± 223	757 ± 38	1086 ± 168
Mg	2446 ± 147	1622 ± 127**	2731 ± 175	1583 ± 201***
Mn	734 ± 105	387 ± 149	543 ± 31	263 ± 78
S	15225 ± 201	12114 ± 876	15071 ± 225	11641 ± 1298
P	14263 ± 202	12123 ± 378**	14676 ± 358	11920 ± 139***

LEAVES				
	wild type		*mpk6*	
Element	Control	2 µM Cu	Control	2 µM Cu
Cu	11 ± 1	11 ± 1	8 ± 1	15 ± 1***
Zn	75 ± 4	81 ± 13	62 ± 3	56 ± 7
K	28620 ± 1162	31132 ± 501	26024 ± 769	26199 ± 1427
Ca	40223 ± 1435	38196 ± 1804	41298 ± 1756	38701 ± 1644
Fe	94 ± 15	96 ± 14	67 ± 2	76 ± 5
Mg	6191 ± 245	6420 ± 290	6511 ± 160	6461 ± 217
Mn	117 ± 5	104 ± 9	121 ± 6	99 ± 4
S	8082 ± 134	8819 ± 633	8682 ± 123	8883 ± 684
P	9584 ± 288	9490 ± 106	9681 ± 222	9017 ± 291

Whereas no differences were observed for other elements in leaves under Cu stress, the amount of K, Mg and P decreased in roots, while Ca concentrations increased by Cu exposure in both wild type and *mpk6* mutant plants (Table 6.1.).

Because leaves of *mpk6* knockout plants display an increased amount of Cu after exposure, which was not seen in wild type plants, expression levels of Cu-binding ligands, metallothioneins (MTs), and phytochelatin (PC) synthase, were analyzed in roots and leaves of Cu-exposed *Arabidopsis* plants. In the roots (Table 6.2.), gene expression of *MT1a* and *MT1c* was differently regulated in wild type plants after Cu exposure. In wild type plants, transcript levels of *MT1a* were already significantly elevated after 2h Cu exposure and increased 9-fold after 24h exposure to Cu. The opposite was observed for the expression of *MT1c* with a significant decrease after 2h Cu exposure and an almost complete inhibition after 24h Cu exposure. In *mpk6* knockouts, no significant differences for these genes were observed. In both genotypes, *PCS1* and *MT2a* were significantly induced after 2h and 24h exposure to Cu respectively.

In leaves (Table 6.2.), no genotypic differences were noticed and the gene expression of both *PCS1* and *MT2a* was induced after 24h of Cu-exposure. When exposed during 1 week, *MT1c* transcript levels were elevated from 5 µM Cu on in the *mpk6* knockout mutant (Table 6.3.).

6.2.3. MPK6 has an effect on the cellular redox status under Cu stress

As MPK6 affects the Cu tolerance of *Arabidopsis* plants, we investigated the role of MPK6 in controlling the cellular redox status after Cu exposure. The cellular redox state was analysed by determining (1) ROS production, (2) oxidative damage to cell membranes indicated by lipid peroxidation, (3) the reduced (GSH) and oxidized (GSSG) levels of glutathione, (4) the activities of antioxidative enzymes and (5) the transcript levels of pro- as well as antioxidative enzymes.

ROS production was estimated by the measurement of H_2O_2 in roots and leaves of Cu-exposed wild type and *mpk6* knockout plants. Roots of Cu-exposed wild type plants accumulated significantly higher H_2O_2 levels after 24h Cu exposure than non-exposed plants (Figure 6.2.A). In *mpk6* mutants, no change of the H_2O_2 content was observed in roots after exposure to Cu. In leaves, no significant differences in H_2O_2 content were found between control and Cu-exposed wild type seedlings (Figure 6.2.B). In contrast, leaves of *mpk6* mutant plants showed a significant increase in H_2O_2 concentration after 24h exposure to Cu.

Table 6.2.: *Transcript levels in roots and leaves of 3-week-old wild type and mpk6 knockout Arabidopsis thaliana plants grown on hydroponics and exposed to 2 µM $CuSO_4$ during 0, 2 or 24h. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype on each time point: downregulation:* p<0.01 | p < 0.001 *; upregulation:* p<0.01 | p < 0.001 *).*

ROOTS										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	wild type	*mpk6*	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
PCS1	1.00±0.03	1.09±0.11	1.18±0.06	6.94±0.21	1.17±0.05	6.89±0.57	1.11±0.03	1.58±0.14	1.21±0.06	1.51±0.09
MT1a	1.00±0.12	0.71±0.20	0.93±0.05	2.86±0.54	1.29±0.25	2.28±0.29	0.90±0.08	8.50±3.44	1.00±0.16	1.69±0.25
MT1c	1.00±0.03	1.47±0.11	1.05±0.17	0.44±0.07	0.85±0.16	0.55±0.11	1.21±0.12	0.14±0.01	0.78±0.11	0.51±0.11
MT2a	1.00±0.22	1.47±0.11	4.18±0.30	3.51±0.70	3.72±0.96	1.92±0.18	1.88±0.26	15.0±1.93	0.90±0.03	9.68±1.07
MT2b	1.00±0.10	1.00±0.11	1.34±0.14	1.30±0.20	0.72±0.06	1.01±0.12	1.18±0.20	1.72±0.20	0.62±0.02	1.31±0.05
MT3	1.00±0.06	1.39±0.11	1.44±0.20	1.11±0.25	0.86±0.12	0.61±0.05	1.29±0.20	1.14±0.09	0.80±0.06	0.89±0.12

LEAVES										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	wild type	*mpk6*	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
PCS1	1.00±0.12	1.13±0.11	1.20±0.08	1.05±0.10	1.40±0.15	1.43±0.11	1.47±0.11	3.56±0.31	1.33±0.17	3.89±0.41
MT1a	1.00±0.23	0.61±0.07	1.04±0.14	1.05±0.10	0.85±0.14	0.84±0.15	0.59±0.03	0.88±0.18	0.88±0.28	0.59±0.08
MT1c	1.00±0.22	1.17±0.18	1.14±0.17	0.52±0.05	1.04±0.11	0.65±0.09	1.42±0.18	0.65±0.14	1.20±0.20	0.70±0.22
MT2a	1.00±0.21	0.95±0.01	0.57±0.04	0.52±0.11	0.52±0.06	0.56±0.03	1.29±0.12	2.53±0.19	1.07±0.11	2.20±0.30
MT2b	1.00±0.17	1.14±0.12	1.29±0.11	1.09±0.05	1.27±0.04	1.19±0.04	1.15±0.06	1.35±0.14	0.94±0.05	0.96±0.15
MT3	1.00±0.21	1.31±0.09	1.41±0.01	1.11±0.28	1.69±0.03	1.74±0.11	1.67±0.09	2.16±0.34	1.33±0.17	1.77±0.18

Table 6.3.: *Transcript levels in leaves of 2-week-old wild type and mpk6 knockout Arabidopsis thaliana plants exposed to different concentrations of $CuSO_4$ during 1 week. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulated:* p<0.01 | p < 0.001 *; upregulated:* p<0.01 | p < 0.001 *).*

LEAVES										
	Control		2 µM Cu		5 µM Cu		10 µM Cu		15 µM Cu	
Gene	wild type	*mpk6*	wild type	*mpk6*	wild type	*mpk6*	wild type	*mpk6*	wild type	*mpk6*
PCS1	1.00±0.17	0.92±0.08	0.97±0.19	1.28±0.11	1.53±0.06	1.88±0.04	1.61±0.14	2.49±0.44	1.64±0.07	1.65±0.18
MT1a	1.00±0.15	0.33±0.01	0.67±0.05	0.35±0.01	0.60±0.01	0.54±0.06	0.63±0.06	0.67±0.09	0.54±0.07	0.53±0.03
MT1c	1.00±0.03	0.37±0.01	0.56±0.05	0.50±0.11	0.73±0.06	1.08±0.09	1.01±0.26	1.16±0.12	0.79±0.11	1.24±0.19
MT2a	1.00±0.09	1.49±0.35	1.46±0.24	1.48±0.17	1.31±0.10	1.50±0.14	1.75±0.18	1.18±0.11	0.84±0.04	0.66±0.03
MT2b	1.00±0.24	0.78±0.12	0.59±0.03	0.60±0.02	0.58±0.04	0.67±0.04	0.55±0.03	0.60±0.04	0.28±0.02	0.23±0.02
MT3	1.00±0.01	0.89±0.06	0.92±0.11	0.89±0.06	0.76±0.01	0.84±0.01	0.74±0.08	0.70±0.03	0.30±0.01	0.34±0.04

As a parameter for the amount of oxidative damage, lipid peroxidation was estimated in roots and leaves of Cu-exposed plants by determining thiobarbituric acid reactive metabolites (TBArm). In roots of both genotypes (Figure 6.2.C), significantly elevated levels of TBArm were observed after 24h exposure to Cu. Although increased TBArm levels were also observed in leaves of wild type plants, leaves of *mpk6* knockout plants showed no differences in TBArm after Cu exposure (Figure 6.2.D).

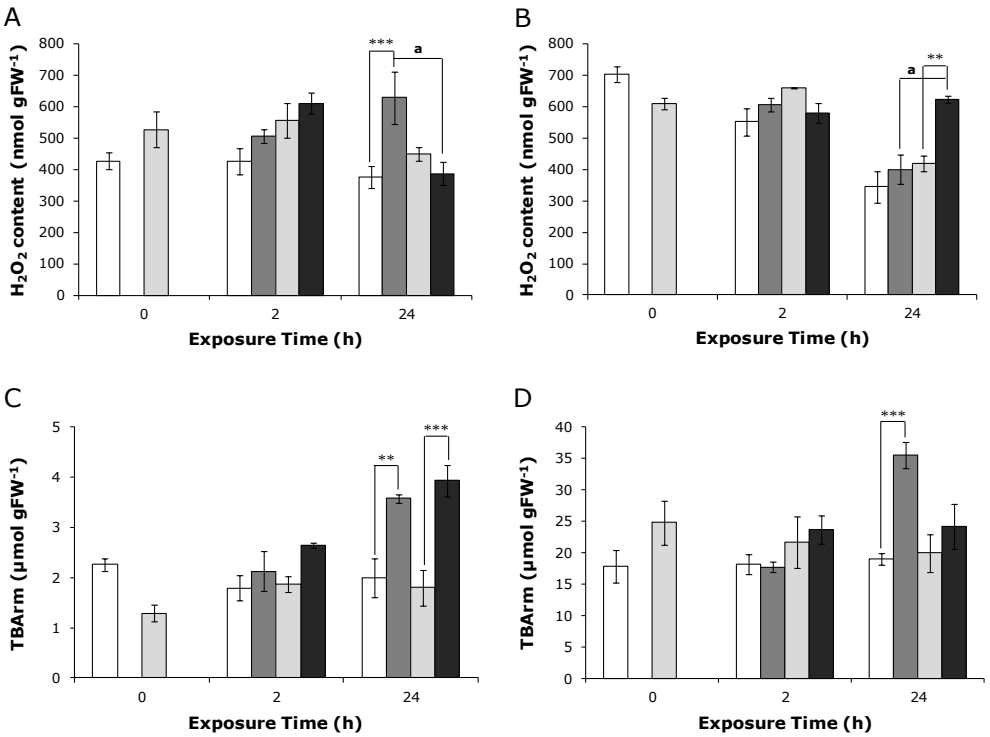

Figure 6.2.: *H_2O_2 content (nmol gFW^{-1}) and lipid peroxidation (µmol gFW^{-1}) in roots (**A**-**C**) and leaves (**B**-**D**) of 3-week-old Arabidopsis thaliana wild type and mpk6 knockout plants grown on hydroponics and exposed to 2 µM $CuSO_4$ during 0, 2 and 24h (□ = wild type non-exposed, ▩ = wild type exposed, □ = mpk6 non-exposed, ■ = mpk6 exposed). Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: **: p < 0.05; ***: p < 0.01; genotype*treatment interaction effect: a: p < 0.01).*

Levels of GSH and GSSG were investigated in roots and leaves of Cu-exposed *Arabidopsis* plants (Table 6.4.). Already after 2h of Cu exposure, the amount of GSH was significantly reduced in roots of wild type and *mpk6* mutant plants. In contrast to wild type seedlings, the concentration of GSSG was also significantly

increased in Cu-exposed *mpk6* knockouts leading to an increased GSSG/GSH ratio. The higher increase in the amount of GSSG in Cu-exposed *mpk6* seedlings compared to Cu-exposed wild type plants is due to a significantly lower GSSG concentration in non-exposed *mpk6* plants at 2h after the start of the day. In roots of both wild type and *mpk6* mutant seedlings exposed to Cu during 24h, the amount of GSH was decreased while the concentration of GSSG was increased, resulting in a significant elevation of the GSSG/GSH ratio. In leaves of Cu-exposed wild type and *mpk6* knockout plants, no significant changes in the cellular redox status were observed. As seen in the roots, the GSSG concentration in non-exposed *mpk6* mutants was lower than in non-exposed wild type plants, which results in a lower GSSG/GSH ratio under control conditions. Nevertheless this was apparent before the start of the day, but this difference disappeared after the onset of light.

Activities of several enzymes involved in antioxidative defence were also examined in roots and leaves of *Arabidopsis* seedlings after Cu exposure (Table 6.5.). The activities of the superoxide dismutases (SOD) were differently affected in roots of wild type and *mpk6* mutant seedlings. The SOD activity was significantly induced in *mpk6* mutants after 2h exposure to Cu. In contrast, SOD levels of wild type plants were not altered after Cu exposure. Guaiacol peroxidase (GPOD), catalase (CAT) and glutathione reductase (GR) activity levels were enhanced in the same way in both wild types and *mpk6* knockouts, with increased activities at 24h after Cu exposure. In the leaves, the activities of peroxidases, determined as SPOD and GPOD, and CAT were significantly increased in leaves of wild type plants after 24h exposure to Cu, whereas no changes in the activity of these enzymes were observed in *mpk6* knockout plants.

Transcript levels of pro- and antioxidative enzymes were measured in roots (Table 6.6.) and leaves (Table 6.7.) of wild type and *mpk6* knockout *Arabidopsis thaliana* plants. Concerning the pro-oxidative side of the redox balance, gene expression of the lipoxygenases *LOX1* and *LOX2* showed no differences between wild type and *mpk6* mutant seedlings. Transcript levels of *LOX1* and *LOX2* were elevated in a similar way in wild type and *mpk6* mutant plants after 2 and 24h of Cu exposure respectively (Table 6.6. and 6.7.).

Table 6.4.: *Concentration (nmol gFW^{-1}) of reduced (GSH) and oxidized (GSSG) glutathione in leaves and roots of 3-week-old Arabidopsis thaliana wild type and mpk6 knockout plants grown on hydroponics and exposed to 2 µM $CuSO_4$ during 0, 2 and 24h (GSSG levels were expressed in GSH equivalents). Values are mean ± S.E. of ± 4 biologically independent replicates (significance level: ***: p < 0.01; genotype effect: b: p < 0.05; a: p < 0.01).*

ROOTS					
		wild type		*mpk6*	
Time	Metabolite	Control	2 µM Cu	Control	2 µM Cu
0h	GSH + GSSG	150.5 ± 14.5		148.5 ± 2.4	
	GSH	147.1 ± 15.0		144.6 ± 1.7	
	GSSG	3.4 ± 1.1		3.9 ± 1.3	
	GSSG /GSH	0.024 ± 0.008		0.027 ± 0.009	
2h	GSH + GSSG	168.6 ± 11.4	62.0 ± 10.5***	150.4 ± 4.6	56.9 ± 2.4***
	GSH	149.7 ± 8.6	55.9 ± 16.5***	149.6 ± 4.8	25.6 ± 4.5***
	GSSG	18.9 ± 7.3	16.8 ± 4.1	0.8 ± 0.2[a]	27.4 ± 1.3***
	GSSG /GSH	0.163 ± 0.039	0.214 ± 0.080	0.006 ± 0.002[a]	1.49 ± 0.56***
24h	GSH + GSSG	148.7 ± 9.8	45.9 ± 4.8***	150.8 ± 5.7	46.6 ± 1.0***
	GSH	134.7 ± 13.7	11.7 ± 1.5***	148.2 ± 5.7	16.4 ± 3.2***
	GSSG	13.9 ± 6.5	30.5 ± 2.1	2.6 ± 0.6	34.8 ± 6.0***
	GSSG /GSH	0.117 ± 0.058	3.04 ± 0.41***	0.014 ± 0.002	3.09 ± 0.58***

LEAVES					
		wild type		*mpk6*	
Time	Metabolite	Control	2 µM Cu	Control	2 µM Cu
0h	GSH + GSSG	379.6 ± 58.2		341.3 ± 51.8	
	GSH	336.6 ± 58.4		316.8 ± 34.9	
	GSSG	43.0 ± 1.6		23.8 ± 3.8[a]	
	GSSG /GSH	0.138 ± 0.021		0.090 ± 0.018	
2h	GSH + GSSG	464.9 ± 41.8	440.5 ± 61.5	404.1 ± 29.5	377.6 ± 23.3
	GSH	391.9 ± 21.3	386.6 ± 49.7	365.6 ± 30.7	343.4 ± 22.9
	GSSG	50.7 ± 10.4	53.8 ± 11.9	47.2 ± 4.0	34.2 ± 3.6
	GSSG /GSH	0.134 ± 0.032	0.135 ± 0.013	0.129 ± 0.005	0.101 ± 0.012
24h	GSH + GSSG	277.3 ± 10.3	398.9 ± 49.2	329.0 ± 35.6	380.5 ± 42.3
	GSH	244.6 ± 25.3	331.2 ± 43.2	304.5 ± 35.9	344.2 ± 46.9
	GSSG	56.9 ± 0.3	67.8 ± 8.1	24.4 ± 4.0[b]	36.4 ± 8.7
	GSSG /GSH	0.260 ± 0.014	0.208 ± 0.018	0.084 ± 0.015[a]	0.119 ± 0.039

Table 6.5.: *Enzyme activities (U gFW^{-1} for SOD, mU gFW^{-1} for all other enzymes) in roots and leaves of 3-week-old Arabidopsis thaliana wild type and mpk6 knockout plants grown on hydroponics and exposed to 2 µM $CuSO_4$ during 0, 2 and 24h. Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: **: p < 0.05; ***: p < 0.01; genotype*treatment interaction effect: a: p < 0.01).*

ROOTS					
		wild type		*mpk6*	
Time	**Enzyme**	**Control**	**2 µM Cu**	**Control**	**2 µM Cu**
0h	**SOD**	555 ± 58		468 ± 66	
	SPOD	10336 ± 483		4988 ± 243	
	GPOD	11752 ± 1328		7632 ± 846	
	GR	309 ± 32		232 ± 28	
	CAT	71 ± 2		65 ± 2	
2h	**SOD**	325 ± 2	368 ± 13	322 ± 16	491 ± 64**
	SPOD	7337 ± 682	7863 ± 371	5946 ± 303	8033 ± 339
	GPOD	8751 ± 837	9733 ± 417	6935 ± 506	9171 ± 940
	GR	265 ± 16	301 ± 16	229 ± 16	298 ± 31
	CAT	59 ± 5	74 ± 4	58 ± 5	73 ± 4
24h	**SOD**	512 ± 65	506 ± 18	542 ± 68	578 ± 29
	SPOD	9556 ± 732	11293 ± 779	8725 ± 494	11063 ± 814
	GPOD	10123 ± 430	21964 ± 731***	8975 ± 475	20913 ± 2091***
	GR	244 ± 5	376 ± 28**	262 ± 11	377 ± 41**
	CAT	59 ± 3	84 ± 5***	62 ± 4	76 ± 5

LEAVES					
		wild type		*mpk6*	
Time	**Enzyme**	**Control**	**2 µM Cu**	**Control**	**2 µM Cu**
0h	**SOD**	1316 ± 131		366 ± 120	
	SPOD	932 ± 156		425 ± 39	
	GPOD	432 ± 38		259 ± 36	
	GR	460 ± 62		781 ± 45	
	CAT	149 ± 8		160 ± 9	
2h	**SOD**	1251 ± 166	1122 ± 176	1380 ± 207	1323 ± 135
	SPOD	1875 ± 83	1616 ± 21	1354 ± 116	1668 ± 97
	GPOD	610 ± 49	589 ± 26	765 ± 62	762 ± 39
	GR	841 ± 37	971 ± 45	901 ± 106	929 ± 90
	CAT	224 ± 38	194 ± 11	157 ± 13	240 ± 36
24h	**SOD**	1490 ± 71	1441 ± 226	1114 ± 106	1312 ± 89
	SPOD	1764 ± 120	2992 ± 242***	1407 ± 125	1696 ± 104[a]
	GPOD	716 ± 45	976 ± 90**	829 ± 38	862 ± 14
	GR	961 ± 40	1035 ± 40	939 ± 44	1008 ± 30
	CAT	217 ± 15	342 ± 36***	185 ± 18	256 ± 10

In roots of Cu-exposed plants, expression of the NADPH oxidases *RBOHC* and *RBOHD* was regulated differently in wild type and *mpk6* knockout seedlings (Table 6.6.). Transcript levels of *RBOHC* were elevated 2-fold in wild type plants after 2h exposure to Cu. In contrast, *mpk6* mutant plants showed no changes in *RBOHC* expression after Cu exposure. Gene expression of *RBOHD* was significantly increased in both wild type and *mpk6* plants after 2h Cu exposure. After 24h exposure to Cu, transcript levels of *RBOHD* were still elevated in wild type plants, whereas *mpk6* knockout plants showed no significant changes in gene expression.

Transcript levels of enzymes involved in the antioxidative defence of the cell were also measured in roots and leaves of Cu-exposed wild type and *mpk6* knockout seedlings. In the roots, genotype-dependent differences in gene expression were observed for the Cu/Zn superoxide dismutases (*CSD1* and *CSD2*) and the catalases (*CAT1* and *CAT3*) (Table 6.6.). In wild type plants, transcript levels of *CSD1* were already significantly elevated after 2h Cu exposure. After 24h exposure to Cu, gene expression of *CSD1* was also significantly increased in *mpk6* mutant seedlings but was lower than the gene expression level of wild type plants. In contrast, expression of *CSD2* was induced in *mpk6* plants after 24h Cu exposure, whereas wild type plants showed no significant changes in *CSD2* levels. Regarding the catalases, gene expression of *CAT1* was significantly elevated in both wild types and *mpk6* knockouts after 2h exposure to Cu. After 24h, transcript levels of *CAT1* were still high in wild type plants, whereas in *mpk6* mutants they were similar to the levels of non-exposed seedlings. In contrast, transcript levels of *CAT3* were induced after 24h exposure to Cu in *mpk6* mutant plants, while *CAT3* expression was not changed in wild type plants. The leaves showed no significant differences in expression of these genes between both genotypes after exposure to Cu with an overall decrease of *CSD2* after 24h (Table 6.7.).

When *Arabidopsis* plants were exposed to Cu during 1 week (Table 6.8.), a strong increase in *CSD1* and *CSD2* transcript levels, concomitant with a strong decrease in *FSD1* transcript levels were observed in leaves of non-exposed *mpk6* knockouts as compared to wild type plants. In contrast, *CSD1* and *CSD2* expression was significantly increased in wild type seedlings after 1 week Cu exposure whereas no changes in gene expression of *CSD1* and *CSD2* were seen

in Cu-exposed *mpk6* mutants as compared to their control conditions. Upon Cu exposure, *FSD1* transcript levels further decreased in leaves of both genotypes. In addition, transcript levels of *CAT2* were significantly decreased in leaves of non-exposed *mpk6* knockout plants. However, after 1 week Cu exposure, *CAT2* transcripts were significantly reduced in Cu-exposed wild types.

6.2.4. MPK6 affects transcript levels via regulation of MAPKinase signalling as well as via miRNA's

Because MPK6 is a signalling molecule able to phosphorylate transcription factors, gene expression of the transcription factors *WRKY22/25/29* and *ZAT12* was determined in roots and leaves of Cu-exposed *mpk6* knockout plants and compared to wild type plants. Expression levels of *MPK3*, a MAPK belonging to the same group as MPK6 and with the same function, were measured after Cu exposure. In the roots, transcript levels of *MPK3* were significantly increased in both wild type and *mpk6* mutant plants after 2h exposure to Cu (Table 6.6.). In contrast, after 24h Cu exposure expression of *MPK3* was still elevated in wild type plants, while *mpk6* knockout plants showed no difference in gene expression. After exposure to Cu, the leaves showed similar gene expression patterns for *MPK3* in wild types and *mpk6* knockouts, with a general increase after 24h (Table 6.7.). However, after 1 week exposure to different Cu concentrations, leaves showed the same response as was seen in the roots; transcript levels of *MPK3* were significantly induced in leaves of Cu-exposed wild type seedlings whereas no changes in *MPK3* expression were observed in *mpk6* mutant plants (Table 6.8.). The roots showed a genotype-dependent regulation of *WRKY22* and *WRKY25* (Table 6.6.) under Cu exposure. Transcript levels of *WRKY22* were 4 times higher in 2h Cu-exposed wild type seedlings as compared to the non-exposed seedlings. In contrast, *mpk6* knockout plants showed no significant increase in gene expression of *WRKY22*. Although wild type and *mpk6* knockout plants showed a similar increase in gene expression of *WRKY25* after 2h exposure to Cu, transcript levels of *WRKY25* were regulated differently after 24h exposure to Cu. In wild type plants, expression of *WRKY25* was significantly elevated after 24h Cu exposure, whereas *mpk6* mutants showed no significant change in transcript levels of *WRKY25*. In addition, *WRKY29* and *ZAT12* showed the same response in gene expression after exposure to Cu in wild type as well

as in *mpk6* mutant seedlings. In the leaves (Table 6.7.), no genotype specific responses were observed. The *WRKY22/25/29* and *ZAT12* transcription factors showed similar gene expression responses after exposure to Cu in wild type and *mpk6* plants, as compared to their respective controls.

Gene expression of *CSD1/2* is known to be regulated by miRNA398. Since differences in root transcript levels of *CSD1/2* were observed between Cu-exposed wild type and *mpk6* knockouts, a possible regulation of miRNA398 by MPK6 was investigated. Therefore, gene expressions of primary transcripts of CSD1/2 and miRNA398 were determined. The primary transcript of CSD1 (*hnCSD1*) showed a similar expression pattern as *CSD1* in roots of both wild type and *mpk6* knockout plants (Table 6.6.). Expression of the primary transcript of CSD2 (*hnCSD2*) was not altered in wild type or *mpk6* mutant seedlings after Cu exposure. In addition, primary transcript levels of the CSD-regulating miRNA398b/c (*pri-MIR398b* and *pri-MIR398c*) were significantly reduced in wild type plants after 2 and 24h exposure to Cu, whereas no changes in *pri-MIR398b* and *pri-MIR398c* were seen in *mpk6* knockout plants (Table 6.6.).

The gene expression regulation of *CSDs* by MPK6, observed in roots of 24h Cu-exposed plants, was also found in leaves of *Arabidopsis* plants exposed to Cu during 1 week with a general increased transcript level (Table 6.8.). Although gene expressions of *CSD1* and *CSD2* were significantly higher in non-exposed *mpk6* plants as compared to wild type plants, transcript levels of *CSD1* and *CSD2* were strongly and significantly induced in leaves of wild type plants after exposure to all Cu concentrations, whereas *CSD1* and *CSD2* transcripts showed no increase in *mpk6* knockout seedlings after Cu exposure. In contrast, expression of the primary transcripts of *CSD1* and *CSD2* did not change after Cu exposure in both wild type and *mpk6* mutant plants and was the same in both non-exposed genotypes. Gene expression of *pri-MIR398b* and *pri-MIR398c* in non-exposed *mpk6* seedlings was significantly reduced in contrast to the non-exposed wild type seedlings. After Cu exposure, expression of *pri-MIR398b* and *pri-MIR398c* transcripts was completely downregulated in wild type plants, comparable to the control levels in *mpk6* mutants, for which the expression was only slightly reduced as compared to control conditions. Transcript levels of *pri-MIR398a* showed the reverse expression pattern.

Table 6.6.: *Transcript levels in roots of 3-week-old wild type and mpk6 knockout Arabidopsis thaliana plants grown on hydroponics and exposed to 2 µM $CuSO_4$ during 0, 2 or 24h. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulation:* p<0.01 | p < 0.001 *; upregulation:* p<0.01 | p < 0.001 *).*

ROOTS										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	**wild type**	***mpk6***	**Control**	**2 µM Cu**	**Control**	**2 µM Cu**	**Control**	**2 µM Cu**	**Control**	**2 µM Cu**
LOX1	1.00±0.06	0.69±0.11	0.95±0.17	6.58±1.92	1.15±0.21	6.83±1.90	1.04±0.18	53.2±21.6	1.02±0.05	13.4±5.21
RBOHC	1.00±0.04	1.09±0.11	0.71±0.05	1.39±0.07	1.28±0.10	1.36±0.13	0.79±0.01	0.59±0.09	1.08±0.12	0.85±0.16
RBOHD	1.00±0.12	0.81±0.11	0.54±0.02	5.01±0.54	0.46±0.03	3.85±0.85	0.62±0.07	2.53±0.59	0.60±0.09	1.87±0.39
CSD1	1.00±0.04	1.09±0.14	0.96±0.04	1.42±0.04	0.95±0.05	1.17±0.12	0.83±0.05	1.70±0.21	0.77±0.08	1.37±0.18
CSD2	1.00±0.14	1.18±0.27	0.88±0.05	1.11±0.12	0.80±0.05	0.88±0.10	1.01±0.12	1.34±0.13	0.74±0.06	1.40±0.25
FSD1	1.00±0.15	2.49±1.31	3.16±0.41	1.05±0.56	1.57±0.09	1.84±0.72	4.95±1.64	0.04±0.01	1.49±0.88	0.09±0.04
APX1	1.00±0.11	1.24±0.11	1.04±0.03	1.49±0.17	1.17±0.08	1.43±0.19	0.95±0.06	1.31±0.08	1.06±0.12	1.30±0.10
CAT1	1.00±0.05	0.88±0.08	1.86±0.25	4.91±0.04	1.41±0.08	3.34±0.20	1.12±0.11	5.19±1.49	0.98±0.11	2.06±0.62
CAT2	1.00±0.07	1.07±0.07	1.14±0.06	0.73±0.03	1.41±0.13	0.86±0.10	1.31±0.04	0.95±0.14	1.53±0.06	1.10±0.06
CAT3	1.00±0.03	1.08±0.11	1.56±0.16	1.71±0.06	1.42±0.02	1.27±0.16	1.48±0.03	1.47±0.06	1.15±0.15	2.07±0.10
MPK3	1.00±0.09	0.79±0.11	0.94±0.07	8.85±1.09	1.02±0.07	6.98±1.18	1.14±0.03	2.45±0.32	1.36±0.13	1.70±0.26
WRKY22	1.00±0.04	0.82±0.11	0.52±0.07	1.91±0.33	0.91±0.20	1.63±0.28	0.56±0.01	0.63±0.07	0.73±0.09	0.69±0.07
WRKY25	1.00±0.06	1.25±0.11	1.16±0.10	7.77±0.82	1.66±0.16	7.43±1.09	1.19±0.08	4.39±0.87	1.46±0.34	3.12±0.20
WRKY29	1.00±0.08	1.07±0.11	1.49±0.35	7.96±0.37	2.55±0.41	8.48±0.52	1.16±0.15	7.10±0.81	1.62±0.10	5.20±0.66
ZAT12	1.00±0.29	0.51±0.11	0.57±0.11	234±60	1.13±0.25	207±67	1.31±0.25	52.3±22.2	1.18±0.29	19.2±3.70
hnCSD1	1.00±0.01	0.80±0.11	0.87±0.05	2.40±0.38	0.95±0.06	2.10±0.29	0.95±0.02	1.76±0.27	0.88±0.04	1.30±0.16
hnCSD2	1.00±0.13	1.05±0.11	1.23±0.07	0.83±0.13	1.08±0.05	0.87±0.08	1.19±0.10	1.92±0.31	1.02±0.09	1.44±0.15
pri-MIR398a	1.00±0.12	1.07±0.11	1.27±0.25	21.6±2.59	1.35±0.37	61.5±18.2	2.25±0.12	11.2±2.24	2.06±0.76	2.93±0.45
pri-MIR398b	1.00±0.08	1.16±0.11	1.43±0.18	0.70±0.13	0.94±0.03	0.60±0.05	1.65±0.33	0.45±0.02	1.25±0.35	0.73±0.09
pri-MIR398c	1.00±0.12	1.11±0.11	1.61±0.20	0.71±0.13	0.94±0.04	0.65±0.07	1.67±0.29	0.47±0.01	1.17±0.30	0.67±0.09

Table 6.7.: *Transcript levels in leaves of 3-week-old wild type and mpk6 knockout Arabidopsis thaliana plants grown on hydroponics and exposed to 2 µM $CuSO_4$ during 0, 2 or 24h. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulated:* p<0.01 | p < 0.001 *;upregulated:* p<0.01 | p < 0.001 *).*

LEAVES										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	wild type	*mpk6*	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu	Control	2 µM Cu
LOX2	1.00±0.09	0.68±0.06	2.53±0.75	1.26±0.30	1.33±0.05	0.80±0.07	0.73±0.06	2.90±0.54	0.82±0.06	2.31±0.21
RBOHC	1.00±0.02	0.94±0.02	1.09±0.11	0.85±0.14	1.00±0.10	0.85±0.01	0.82±0.08	1.71±0.16	0.78±0.03	1.68±0.33
CSD1	1.00±0.10	1.07±0.08	1.19±0.12	0.98±0.14	1.20±0.12	1.26±0.08	1.28±0.05	1.54±0.03	1.04±0.12	1.42±0.18
CSD2	1.00±0.09	1.61±0.21	1.60±0.23	1.18±0.23	1.64±0.12	1.74±0.16	1.56±0.09	1.06±0.08	1.63±0.04	0.93±0.10
FSD1	1.00±0.60	1.25±0.63	0.58±0.18	0.14±0.06	0.45±0.04	0.28±0.13	0.27±0.05	0.12±0.05	0.20±0.05	0.05±0.01
APX1	1.00±0.01	1.41±0.10	1.51±0.19	1.22±0.05	1.18±0.25	1.12±0.03	1.92±0.10	1.85±0.05	1.26±0.01	1.23±0.16
CAT1	1.00±0.20	1.15±0.10	4.52±0.69	3.17±0.67	3.95±0.82	3.15±0.19	2.36±0.13	1.74±0.22	1.50±0.37	1.49±0.10
CAT2	1.00±0.07	1.26±0.09	0.44±0.03	0.36±0.05	0.48±0.05	0.37±0.03	0.98±0.08	1.06±0.08	1.08±0.06	1.23±0.09
CAT3	1.00±0.08	1.22±0.17	3.69±0.46	2.68±0.10	2.99±0.47	3.52±0.33	1.81±0.28	2.59±0.52	1.49±0.29	2.09±0.34
MPK3	1.00±0.02	0.98±0.10	1.03±0.07	1.06±0.11	1.09±0.14	1.21±0.07	1.02±0.16	3.06±0.16	1.25±0.16	4.81±0.73
WRKY22	1.00±0.20	1.15±0.10	0.88±0.08	0.53±0.06	0.98±0.12	0.48±0.06	0.91±0.18	0.97±0.06	1.22±0.08	1.11±0.06
WRKY25	1.00±0.12	1.00±0.17	1.63±0.44	1.20±0.11	2.45±0.39	1.92±0.30	2.19±0.17	5.09±0.42	1.03±0.14	8.61±0.84
WRKY29	1.00±0.21	1.38±0.12	1.97±0.12	1.23±0.15	2.48±0.46	1.23±0.12	0.99±0.21	0.86±0.05	1.20±0.08	0.97±0.15
ZAT12	1.00±0.24	2.05±0.57	3.33±1.52	1.20±0.22	1.30±0.34	1.49±0.77	7.50±5.18	2.67±1.12	1.06±0.45	6.64±2.52
hnCSD1	1.00±0.04	0.94±0.03	1.23±0.07	0.97±0.17	1.07±0.08	0.95±0.07	0.99±0.14	1.34±0.06	1.00±0.05	1.53±0.03
hnCSD2	1.00±0.03	1.08±0.05	0.86±0.07	0.69±0.19	0.67±0.11	0.63±0.02	0.81±0.06	0.69±0.08	0.91±0.02	0.48±0.04
pri-MIR398a	1.00±0.01	1.35±0.22	1.11±0.22	1.87±0.03	1.53±0.23	3.17±1.28	1.90±0.29	6.42±1.36	1.58±0.23	5.67±0.95
pri-MIR398b	1.00±0.28	1.06±0.46	0.77±0.10	0.41±0.01	0.47±0.09	0.50±0.08	1.52±0.30	0.41±0.09	1.37±0.14	0.17±0.02
pri-MIR398c	1.00±0.31	1.16±0.53	0.76±0.10	0.30±0.01	0.47±0.05	0.42±0.08	1.50±0.26	0.51±0.18	1.37±0.19	0.13±0.01

Table 6.8.: *Transcript levels in leaves of 2-week-old wild type and mpk6 knockout Arabidopsis thaliana plants exposed to different concentrations $CuSO_4$ during 1 week. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype): downregulated:* p<0.01 | p < 0.001 *;upregulated:* p<0.01 | p < 0.001 *).*

LEAVES										
	Control		2 µM Cu		5 µM Cu		10 µM Cu		15 µM Cu	
Gene	wild type	*mpk6*	wild type	*mpk6*	wild type	*mpk6*	wild type	*mpk6*	wild type	*mpk6*
LOX2	1.00±0.13	0.93±0.07	1.15±0.55	1.18±0.03	4.63±1.12	1.65±0.15	2.76±0.17	2.45±0.07	3.01±0.23	1.61±0.34
RBOHC	1.00±0.43	1.24±0.17	2.26±0.37	1.35±0.02	1.92±0.44	1.62±0.05	1.15±0.29	1.90±0.07	1.14±0.15	0.96±0.18
RBOHD	1.00±0.13	1.02±0.07	1.35±0.12	1.22±0.06	1.45±0.14	1.47±0.02	1.25±0.17	1.39±0.10	0.91±0.09	1.09±0.03
CSD1	1.00±0.28	12.9±0.90	13.9±1.02	17.5±1.41	16.6±1.39	20.9±3.03	17.8±1.50	14.1±1.08	11.3±1.12	7.12±0.49
CSD2	1.00±0.30	8.92±1.13	12.8±1.89	13.1±1.40	14.3±1.51	23.6±6.47	20.4±3.00	18.7±3.29	12.4±1.10	10.2±0.63
FSD1	1.00±0.12	3.76×10^{-3}	1.61×10^{-4}	1.25×10^{-4}	4.52×10^{-5}	1.34×10^{-4}	2.99×10^{-5}	10.4×10^{-4}	1.31×10^{-5}	5.51×10^{-6}
APX1	1.00±0.16	1.98±0.11	0.99±0.04	1.85±0.13	0.73±0.01	1.68±0.21	0.54±0.05	1.18±0.08	0.11±0.03	0.23±0.03
CAT1	1.00±0.23	0.90±0.03	0.87±0.03	0.86±0.11	0.77±0.12	1.01±0.04	0.62±0.02	0.73±0.03	0.45±0.08	0.33±0.03
CAT2	1.00±0.06	0.62±0.07	0.44±0.07	0.78±0.09	0.53±0.02	0.75±0.07	0.59±0.04	1.01±0.07	0.32±0.05	0.73±0.02
CAT3	1.00±0.14	1.33±0.08	1.55±0.33	1.19±0.13	1.27±0.13	1.06±0.19	0.92±0.08	1.14±0.18	0.31±0.03	0.55±0.07
OXI1	1.00±0.12	0.33±0.05	0.92±0.18	0.50±0.09	1.88±0.18	0.55±0.07	2.00±0.34	0.87±0.14	4.07±0.20	1.96±0.18
MPK3	1.00±0.02	1.48±0.09	2.08±0.04	1.60±0.07	1.99±0.21	2.14±0.26	1.84±0.23	1.80±0.19	1.95±0.20	1.65±0.14
hnCSD1	1.00±0.09	0.77±0.01	1.22±0.04	0.85±0.04	1.25±0.11	1.17±0.06	0.95±0.11	1.23±0.08	0.54±0.03	0.69±0.05
hnCSD2	1.00±0.24	0.76±0.02	2.10±0.01	1.40±0.25	1.96±0.16	1.35±0.33	1.93±0.15	1.44±0.04	1.56±0.19	1.14±0.10
pri-MIR398a	1.00±0.42	47.6±3.98	48.4±16.1	63.1±20.4	30.9±6.13	78.5±14.7	93.0±27.1	80.2±10.5	86.3±13.4	96.5±15.8
pri-MIR398b	1.00±0.04	8.05×10^{-3}	2.02×10^{-3}	2.33×10^{-3}	1.63×10^{-3}	1.67×10^{-3}	2.26×10^{-3}	1.51×10^{-3}	1.45×10^{-3}	1.80×10^{-3}
pri-MIR398c	1.00±0.05	4.76×10^{-3}	1.53×10^{-3}	1.58×10^{-3}	1.48×10^{-3}	1.56×10^{-3}	1.18×10^{-3}	1.34×10^{-3}	8.58×10^{-4}	1.23×10^{-3}

6.3. Discussion

In the present study, *mpk6* mutants are more Cu tolerant than wild type plants, based on primary root growth analysis (Figure 6.1.). Nevertheless, after short Cu exposure (24h) in a hydroponics set-up, no differences in fresh weight were observed between both genotypes. As Cu-induced oxidative stress is a basic mechanism by which Cu induces toxicity symptoms in plants (Cuypers *et al.* 2011b), it was investigated whether this may be underlying the Cu tolerance in *mpk6* mutants. For this purpose, 3-week-old wild type and *mpk6* knockout seedlings were exposed to 2 µM Cu during 2 and 24h. First, the concentrations of Cu as well as of other essential elements were determined in leaves and roots of 24h Cu-exposed plants (Table 6.1.). In the roots no difference in Cu content between non-exposed wild type and non-exposed *mpk6* mutant plants was observed. Moreover, after Cu exposure, the roots of both genotypes contained similar amounts of Cu. In contrast, leaves of *mpk6* plants showed a significant increase of Cu concentration in their leaves after Cu exposure, which was not observed in wild type plants. Nevertheless, higher accumulation and consequently storage of Cu in the leaves cannot account for the higher Cu tolerance seen in root growth of *mpk6* mutants exposed to Cu during 1 week (Figure 6.1.).

Once Cu is taken up by the root cells, it is bound to chelators, such as PCs, MTs, organic acids or amino acids, which sequester free metal ions in the cytosol or in subcellular compartments (Clemens 2001, Haydon and Cobbett 2007). They can also be bound to chaperones, which deliver metal ions to organelles and metal-requiring proteins (Grotz and Guerinot 2006, Puig *et al*. 2007). Different authors reported that MT gene expression is strongly induced upon Cu exposure (Zhou and Goldsbrough 1994, Hsieh *et al*. 1995, Roosens *et al*. 2004, Guo *et al*. 2008), whereas the role of PCs in Cu detoxification is not unambiguously demonstrated (Cobbett and Goldsbrough 2002). Because MPK6 regulates downstream gene expression, we examined if MPK6 is involved in expression of genes involved in chelation of excess Cu. Therefore, gene expressions of MT and PC synthases (PCS) were determined in wild type and *mpk6* knockout plants after exposure to Cu. In the roots, expression of *MT1a* increased in wild type plants after exposure to Cu, while *MT1c* expression was reduced (Table 6.2.). Of the different isoforms of metallothioneins, *MT1a* and *MT1c* are the most abundantly expressed in the

roots (Guo *et al*. 2003). Consequently, their contrasting gene regulation may compensate each other's function. In addition, transcript levels of *PCS1* were elevated in roots of both wild type and *mpk6* mutant plants after 2h Cu exposure (Table 6.2.), indicating a role for PCs in the chelation of excess Cu. This was also demonstrated by the decrease of GSH levels observed in Cu-exposed roots (Table 6.4.) in both genotypes. The use of GSH for chelation purposes can result in a depletion of GSH stores, thereby affecting the antioxidative properties of GSH resulting in oxidative stress (Seth *et al*. 2012).

It's well described that Cu-exposure affects the cellular redox state in different plant species (Cuypers *et al*. 2000, 2005, Smeets *et al*. 2009). To obtain a general picture of the cellular redox status in wild type and *mpk6* knockout plants after Cu exposure, the amount of ROS production, the degree of membrane damage and the activity and transcript level of pro- and antioxidative enzymes, were estimated in roots and leaves of wild type and *mpk6* knockout plants. In roots, the production of H_2O_2 was increased in wild type plants after 24h Cu exposure (Figure 6.2.A). In addition to its direct metal-catalysed production, this increase in H_2O_2 content can be explained by the production of H_2O_2 by NADPH oxidases. These enzymes produce superoxide anions, which form H_2O_2 after dismutation (Torres and Dangl 2005). Indeed, transcript levels of *RBOHC* and *RBOHD* were elevated in wild type plants after 2 and 24h Cu exposure (Table 6.6.). As a result, enzyme activities of CAT and GPOD were elevated to neutralize the H_2O_2 produced by the Cu exposure (Table 6.5.). The increase in CAT activity was accompanied by an increase in *CAT1* gene expression (Table 6.6.). Whereas H_2O_2 is not a free radical, it can be converted to the more damaging hydroxyl radical ($^{\bullet}OH$) by means of the Fenton and Haber-Weiss reactions (Kehrer 2000). The hydroxyl radical can react with a variety of cellular components like DNA, proteins and other small molecules. Lipid peroxidation, as a measure of cellular damage caused by the produced ROS, was increased in roots of Cu-exposed wild types and *mpk6* mutants after 24h (Figure 6.2.C). Therefore, a lower root oxidative damage cannot account for the increased Cu tolerance observed for primary root growth. Moreover, already after 2h Cu exposure, GSSG levels, which showed a sharp decrease under non-exposed conditions in comparison to wild type plants, were significantly elevated

in *mpk6* mutant roots indicative of a more oxidized redox status compared to Cu-exposed wild type roots (Table 6.4.). A more oxidized cellular redox environment (GSSG/GSH ratio) was also observed in roots of *Arabidopsis* seedlings exposed to 2 or 5 µM Cu during 24h (Cuypers *et al.* 2011a). In our study this was also observed for both genotypes after 24h and an increased activity of GR (Table 6.5.) could not reduce GSSG efficiently back to GSH as compared to control conditions. The finding that roots of *mpk6* knockout seedlings develop oxidative stress faster than wild type plants seems to contrast with the increased Cu tolerance earlier observed in *mpk6* mutants (Figure 6.1.). Then, the question arises to the function of this early oxidative stress signals. Is it used for root-to-shoot signalling, thereby inducing acclimation responses to long-term metal exposure? However, more research on oxidative stress parameters is needed between 2 and 24h Cu exposure as well as long-term exposure, to investigate the role of MPK6 in controlling the cellular redox status, with specific emphasis on the regulation of GSSG levels by MPK6 and the circadian rhythm.

Because MPK6 is a signalling molecule, able to regulate the expression of target genes via phosphorylation and activation of transcription factors, the gene expression of certain transcription factors as well as enzymes involved in the pro- or antioxidative machinery of the cell was determined. In the roots, transcript levels of the MPK6 homolog *MPK3* were enhanced in both wild type and *mpk6* mutant plants after 2h exposure to Cu (Table 6.6.). After 24h exposure to increased Cu concentrations, *MPK3* expression was not longer increased in *mpk6* mutant seedlings in contrast to wild type plants. Exactly the same regulation was observed for the gene expression of the transcription factor *WRKY25* (Table 6.6.). These observations suggest that after 24h of Cu exposure, MPK6 is responsible for keeping *MPK3* and *WRKY25* transcript levels high. Previously, it was shown that in roots, *MPK3* and *WRKY25* are coregulated upon Cu exposure and that this forms a larger cluster with MPK6 (Opdenakker *et al.* 2012a; chapter 4). Furthermore, expression of *WRKY22* was induced in wild type plants after 2h exposure to Cu but showed no change in transcript levels in *mpk6* mutant plants (Table 6.6.). So, Cu-dependent upregulation of *WRKY22* expression in roots of *Arabidopsis thaliana* is most likely mediated by MPK6. Involvement of WRKY22 was earlier described in other stresses (Asai *et al*.

2002) as well as in metal stress, where it is suggested to be a metal-specific transcription factor as it was involved in Cu responses, but not in Cd responses (Opdenakker *et al.* 2012a; chapter 4). Moreover, a coregulation of *WRKY22* and *RBOHC* in *Arabidopsis* seedlings kinetically exposed to 2 µM Cu, was observed that is also confirmed in the present study. Although WRKY22 and WRKY29 were thought to function in the same stress responses because they share very similar protein structures (Eulgem *et al*. 2000), *WRKY29* gene expression was regulated differently and independently of MPK6 in case of metal stress. Via this MPK6-specific regulation of transcription factors, MPK6 can specifically regulate target genes involved in the production or scavenging of ROS in the cell after Cu exposure.

Besides activation of gene expression, also posttranscriptional regulation is important in the steady state level of gene transcripts. As such, gene expression of *CSDs* in *Arabidopsis* is known to be regulated by miRNA398. Sunkar *et al*. (2006, 2007) revealed a mechanism of posttranscriptional gene regulation in which mRNA of *CSD1* and *CSD2* is degraded by miRNA398b/c, leading to repression of the translation of *CSD1* and *CSD2* transcripts. Cuypers *et al*. (2011a) demonstrated that exposure of *Arabidopsis* seedlings to environmentally realistic Cu concentrations during 24h also decreased levels of mature miRNA398b/c, which in turn led to induced *CSD1* and *CSD2* transcript levels. Furthermore, Yamasaki *et al*. (2009) reported that the transcription factor SPL7 is essential for the response of miRNA398 to Cu. Because we showed that *CSD1* and *CSD2* transcription in roots of *Arabidopsis* seems to be regulated by MPK6, we investigated if this MPK6-mediated gene expression of *CSDs* involves miRNAs. Therefore, we measured gene expression of the primary transcripts of *CSD1*, *CSD2* and the three gene family members of *miRNA398*. In roots of wild type plants, expression of *CSD1* and its primary transcript *hnCSD1* is upregulated after 2 and 24h Cu exposure, while gene expression of *pri-MIR398b* and *pri-MIR398c* was downregulated (Table 6.6.). In contrast, *hnCSD1* expression was elevated in *mpk6* knockout plants after 2h Cu exposure, but *CSD1* transcript levels were not increased. This means that *CSD1* transcripts are degraded by miRNA398 and are not compensated by an increased transcription activity. Indeed, expression of *pri-MIR398b* and *pri-MIR398c* was not downregulated after Cu exposure in *mpk6* mutants. These data suggest that the

SPL7 and miRNA398 regulated expression of *CSD1* seen after Cu exposure is mediated by MPK6.

The Cu content of Cu-exposed *mpk6* mutant leaves was twice as high as in the leaves of non-exposed *mpk6* knockout seedlings. Although the amount of Cu did not significantly differ between leaves of wild type and *mpk6* mutant plants after 24h Cu exposure, no increase in lipid peroxidation was observed in the leaves of *mpk6* mutants as compared to wild type plants. This indicates that *mpk6* mutants experience less oxidative damage upon Cu exposure in the leaves. To further unravel this difference, oxidative stress parameters were investigated in the leaves. *Mpk6* mutant seedlings showed an accumulation of H_2O_2 in their leaves after exposure to Cu (Figure 6.2.B), but no membrane damage, suggesting oxidative signalling. On the other hand, H_2O_2 levels in Cu-exposed wild type seedlings did not change, but oxidative damage occurred. Also activities of H_2O_2 neutralizing enzymes were enhanced in wild type plants indicating a clear stress effect (Table 6.5.).

Although Cu exposure of the leaves resulted in altered gene expression levels of both signalling components and pro- or antioxidative enzymes (Table 6.7.), no involvement of MPK6 was identified after short-term (2 or 24h) Cu exposure. However, after longer exposure times (1 week), MPK6 was observed to specifically regulate transcript levels of the MAPKinase *MPK3*, the pro-oxidative lipoxygenase *LOX2* and the antioxidative defence enzymes *CAT2*, *CSD1* and *CSD2* (Table 6.8.). As earlier demonstrated for the roots, the MPK6-dependent induction of *CSD1/2* expression in Cu-exposed leaves was mediated by miRNA398. In wild type leaves, transcript levels of *pri-MIR398b/c* were completely reduced after Cu exposure leading to a strong increase in *CSD1/2* gene expression (Table 6.8.). In contrast, *mpk6* knockout seedlings showed already under non-exposed conditions low levels of *pri-MIR398b/c* and high amounts of *CSD1/2* transcripts, which were not drastically changed after Cu exposure. All together, we demonstrated that SPL7- and miRNA398-mediated regulation of *CSD* transcript levels can be regulated by MPK6 in *Arabidopsis* plants after Cu exposure.

In conclusion, we showed that MPK6 influences the Cu accumulation in the leaves in Cu-exposed *Arabidopsis* plants. In addition, after Cu exposure, roots of *mpk6* knockout seedlings seem to experience oxidative stress earlier than observed for wild type roots. It is hypothesized that this early oxidative burst is used for root-to-shoot signalling, inducing acclimation responses to Cu exposure. In accordance, leaves of *mpk6* mutants did not suffer lipid peroxidation after Cu exposure in contrast to wild type plants that experienced oxidative membrane damage. In addition, downstream regulation of MPK6 was affected in roots after Cu exposure, revealing transcription factors *WRKY22* and *WRKY25* as downstream targets of MPK6 under Cu stress. Furthermore, previously discovered clusters (*MPK3/WRKY25-MPK6* and *WRKY22/RBOHC*) were emphasized. Whereas in leaves after short term exposure no differences were observed, after prolonged Cu exposure, MPK6 seems to be involved in the SPL7- and miRNA398- mediated regulation of *CSD1/2* transcripts, as was earlier demonstrated in roots of Cu-exposed *Arabidopsis thaliana*.

Chapter 7

The protein kinase MPK6 interferes with the GSH redox status and mediates Cd-induced oxidative stress in *Arabidopsis thaliana*

Kelly Opdenakker, Tony Remans, Heribert Hirt, Jaco Vangronsveld, Ann Cuypers (2012). The protein kinase MPK6 interferes with the GSH redox status and mediates Cd-induced oxidative stress in *Arabidopsis thaliana*. In preparation.

Abstract

The mitogen-activated protein kinase MPK6 is a signalling molecule able to regulate transcription of target genes via the phosphorylation of transcription factors. Activation of MPK6 is mediated by reactive oxygen species (ROS) and is associated with Cd toxicity. In this study, the involvement of MPK6 in Cd-induced oxidative stress was investigated by comparing oxidative stress-mediated parameters between Cd-exposed wild type and *mpk6* knockout *Arabidopsis* seedlings. Although *mpk6* mutants displayed lower concentrations of GSSG under non-exposed conditions, GSH was in both genotypes used for chelation of Cd and in the antioxidative defence. As a consequence of the lower GSSG levels, *mpk6* mutant plants seem to recover much more slowly from the depletion of their GSH pool. Use of GSH for metal chelation can temporary reduce the antioxidative capacity of GSH, causing oxidative stress at the cellular level. The induced oxidative stress is mainly situated at the transcriptional level. After exposure to Cd, MPK6-induced transcription of the NADPH oxidase *RBOHD* in roots of *Arabidopsis* plants via the transcription factors WRKY25 and ZAT12. In Cd-exposed leaves, MPK6 regulates transcript levels of the transcription factor *WRKY22* and the Cu/Zn superoxide dismutases *CSD1/2*.

7.1. Introduction

Cadmium (Cd) can occur in the environment as a consequence of natural sources, mining and industrial processes or the agricultural use of phosphate fertilizers (Vangronsveld *et al*. 1995, Kirkham 2006, Chary *et al*. 2008). Cadmium is a non-essential element for plants and therefore toxic, even at low concentrations. In plants, Cd disrupts physiological processes, resulting in leaf chlorosis and growth inhibition (DalCorso *et al*. 2008, Cuypers *et al*. 2009). At the cellular level, Cd induces oxidative stress, which is a disturbance of the cellular redox status in favour of the pro-oxidants (Smeets *et al*. 2009, Cuypers *et al*. 2011a). The produced reactive oxygen species (ROS) are not always harmful for the cell, they can also act as signalling molecules by transducing signals from a receptor at the plasma membrane to the nucleus. In this way, ROS are involved in the activation of mitogen-activated protein kinase (MAPK) pathways, which play a role in the transcriptional regulation of target genes (Mittler *et al*. 2004, Apel and Hirt 2004).

One of the most studied *Arabidopsis* MAPKinases today is MPK6. MPK6 is a MAPKinase belonging to group A (Ichimura *et al*. 2002) involved in environmental stress (cold, salt, bacterial pathogens) and hormonal responses (ethylene, jasmonic acid) (reviewed in Opdenakker *et al*. 2012b; chapter 1). Activation of MPK6 can be mediated by ROS (Kovtun *et al*. 2000, Rentel *et al*. 2004) and is also implicated in Cd toxicity. Liu *et al*. (2010) demonstrated that the activity of MPK6 was induced by ROS after short-term exposure (less than 1h) to low $CdCl_2$ concentrations (1 µM), initiated by ROS accumulation. In addition, Opdenakker *et al*. (2012a; chapter 4) observed that transcript levels of *MPK6* were increased in roots of *Arabidopsis* plants exposed to 5 µM $CdSO_4$ during 24h. Also in *Medicago sativa* roots as well as in rice roots, the orthologues of *Arabidopsis* MPK6, SIMK and OsMPK6, were activated after exposure to excess Cd ions (Jonak *et al*. 2004, Yeh *et al*. 2007).

MPK6 regulates the expression of target genes via the activation or repression of transcription factors. For example, MPK6 activated by flagellin signalling-induced expression of defence genes via the WRKY transcription factors, WRKY22 and WRKY29 (Asai *et al*. 2002). Transcript levels of *WRKY25*, *WRKY29* and *ZAT12* were elevated in leaves and roots of Cd-exposed *Arabidopsis* plants, suggesting

that these transcription factors play an important role in regulation of the stress response upon Cd exposure (Opdenakker *et al*. 2012a; chapter 4).

WRKY's and ZAT12 transcription factors influence the gene expression of enzymes involved in the control of the cellular redox status. Li *et al*. (2009) reported that WRKY25 is involved in the transcriptional regulation of the antioxidative ascorbate peroxidases *APX1* and *APX2* under heat stress. In studies with *ZAT12* overexpressing as well as knockout plants, it was observed that ZAT12 is involved in the regulation of antioxidative as well as pro-oxidative enzymes, such as superoxide dismutases (*CSD1*, *CSD2*, *FSD1*), peroxidases (*APX1*), NADPH oxidases (*RBOHD*) and lipoxygenases (*LOX4*) (Rhizhsky *et al*. 2004, Davletova *et al*. 2005a-b, Vogel *et al*. 2005).

Shortly summarized, MPK6 is activated by Cd, possibly via the production of ROS, and is able to regulate the gene expression of pro- and antioxidative enzymes via activation of transcription factors. Based on these two findings, we hypothesize that MPK6 is involved in the regulation of the cellular redox status in Cd-exposed *Arabidopsis thaliana* plants. To investigate this, we exposed *Arabidopsis* wild type as well as *mpk6* knockout plants to a sublethal Cd concentration and investigated the effect of the loss of MPK6 on different levels of the cellular redox status.

7.2. Results

7.2.1. MPK6 does not alter element concentrations after Cd exposure

The concentration of Cd and essential elements was determined in roots and leaves of 3-week-old wild type and *mpk6* knockout *Arabidopsis* seedlings after 24h exposure to 5 µM Cd (Table 7.1.). Roots of Cd-exposed wild type and *mpk6* knockout *Arabidopsis* seedlings showed a significant increase in Cd content. Although the increase in Cd levels seemed to be higher in wild type plants than in *mpk6* mutants, no significant genotype*treatment interaction effect was observed. Concentrations of other essential elements were not affected in the roots by Cd exposure. In leaves, the amount of Cd was significantly elevated after Cd exposure in both wild type and *mpk6* mutant plants. Due to the accumulation of Cd in the leaves, Cu levels were significantly decreased after Cd exposure in *mpk6* knockout seedlings in contrast to wild type plants. In addition,

Cd exposure affected the concentration of Mn in the leaves, which was significantly decreased in both wild type and *mpk6* mutant plants.

Table 7.1.: *Element concentrations (mg kgDW^{-1}) in roots and leaves of 3-week-old Arabidopsis wild type and mpk6 knockout seedlings exposed to 5 µM $CdSO_4$ during 24h. Values are mean ± S.E. of at least 5 biologically independent replicates (significance levels: treatment effect: ***: p < 0.01, **: p < 0.05).*

ROOTS				
	wild type		***mpk6***	
Element	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**
Cd	ND	1309 ± 300***	ND	789 ± 168***
Cu	27 ± 2	38 ± 6	22 ± 0.8	28 ± 1
Zn	442 ± 52	511 ± 112	336 ± 33	300 ± 45
K	60662 ± 1372	55268 ± 5906	63568 ± 1947	59408 ± 4584
Ca	2203 ± 210	2626 ± 66	2137 ± 218	1919 ± 78
Fe	695 ± 89	826 ± 43	757 ± 38	684 ± 24
Mg	2446 ± 147	2563 ± 125	2731 ± 175	2524 ± 127
Mn	734 ± 105	781 ± 78	543 ± 31	659 ± 91
S	15225 ± 201	14613 ± 766	15071 ± 225	16067 ± 1166
P	14263 ± 202	14757 ± 359	14676 ± 358	14613 ± 701

LEAVES				
	wild type		***mpk6***	
Element	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**
Cd	ND	267 ± 75***	ND	298 ± 57***
Cu	11 ± 1	8.0 ± 0.6	7.7 ± 0.3	6.2 ± 0.3***
Zn	75 ± 4	73 ± 10	62 ± 3	56 ± 5
K	28620 ± 1162	28763 ± 1253	26024 ± 769	27707 ± 1175
Ca	40223 ± 1435	38347 ± 1043	41298 ± 1756	41522 ± 2113
Fe	94 ± 15	69 ± 4	67 ± 2	63 ± 1
Mg	6191 ± 245	6180 ± 338	6511 ± 160	6633 ± 257
Mn	117 ± 5	80 ± 8**	121 ± 6	86 ± 8**
S	8082 ± 134	9246 ± 322	8682 ± 123	9622 ± 248
P	9584 ± 288	8811 ± 713	9681 ± 222	9215 ± 420

Elevated Cd concentrations can damage cellular components in the plant. Therefore, chelation and sequestration of Cd ions by phytochelatins (PCs) and metallothioneins (MTs) is an important mechanism for detoxification of Cd. Consequently, gene expression of MTs and PC synthases (PCS) was evaluated in roots and leaves of Cd-exposed wild type and *mpk6* mutant seedlings. In roots (Table 7.2.), transcript levels of *MT1a* and *MT1c* were respectively up- or downregulated in wild type seedlings after 24h exposure to Cd. In contrast, *MT1a* and *MT1c* expression was not altered in *mpk6* knockout plants after Cd exposure. In addition, *MT2a* gene expression was significantly elevated in *mpk6* mutants after 2 and 24h Cd exposure whereas wild type plants showed only an increasing trend in *MT2a* levels after 24h exposure to Cd. Additionally, transcript levels of *MT3* were significantly reduced in wild type plants after 24h exposure to Cd, but were not changed in *mpk6* knockouts. In leaves, no differences in gene expression were seen between wild type and *mpk6* knockout plants (Table 7.2.). Transcript levels of phytochelatin synthase (*PCS1*) and metallothionein 2 (*MT2a*) were significantly elevated after 24h exposure to Cd in both wild type and *mpk6* mutant plants.

7.2.2. MPK6 and the cellular redox status after Cd exposure

Exposure to Cd initiates oxidative stress in roots and leaves of *Arabidopsis* plants and activity as well as transcript levels of MPK6 were elevated after Cd exposure (Cuypers *et al*. 2011a, Liu *et al*. 2010). Therefore, we investigated if MPK6 is involved in controlling the cellular redox status after Cd exposure. This was analyzed by measuring the (1) ROS production, (2) oxidative damage to cell membranes, (3) the content of reduced (GSH) and oxidized (GSSG) glutathione and (4) activities and transcript levels of antioxidative enzymes.

Table 7.2.: *Transcript levels in roots and leaves of 3-week-old wild type and mpk6 knockout Arabidopsis thaliana plants exposed to 5 µM $CdSO_4$ during 0, 2 or 24h. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype on each time point: downregulated:* p<0.01 | p < 0.001 *; upregulated:* p<0.01 | p < 0.001 *).*

ROOTS										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	wild type	*mpk6*	Control	5 µM Cd	Control	5 µM Cd	Control	5 µM Cd	Control	5 µM Cd
PCS1	1.00±0.03	1.23±0.06	1.26±0.07	1.90±0.33	1.45±0.07	1.22±0.13	1.33±0.12	1.93±0.23	1.41±0.01	1.88±0.14
MT1a	1.00±0.15	1.03±0.12	0.95±0.16	0.84±0.13	1.07±0.17	0.91±0.13	0.93±0.12	1.41±0.08	0.90±0.06	1.09±0.04
MT1c	1.00±0.21	1.02±0.09	1.20±0.25	0.78±0.18	0.84±0.01	0.89±0.08	2.30±0.34	0.70±0.09	1.49±0.11	1.13±0.12
MT2a	1.00±0.25	2.17±0.39	0.36±0.03	0.47±0.08	0.60±0.09	1.56±0.35	1.18±0.20	1.84±0.08	1.30±0.48	2.63±0.10
MT2b	1.00±0.05	0.60±0.06	0.97±0.09	1.03±0.11	0.72±0.02	0.80±0.07	1.31±0.10	1.43±0.05	0.73±0.06	0.84±0.14
MT3	1.00±0.25	0.89±0.09	1.04±0.23	0.96±0.17	0.76±0.04	1.05±0.19	1.40±0.15	0.81±0.15	0.87±0.07	0.72±0.11

LEAVES										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	wild type	*mpk6*	Control	5 µM Cd	Control	5 µM Cd	Control	5 µM Cd	Control	5 µM Cd
PCS1	1.00±0.04	1.12±0.10	1.41±0.08	1.79±0.13	1.66±0.17	1.57±0.12	1.46±0.13	4.35±0.90	1.45±0.17	4.00±0.58
MT1a	1.00±0.07	0.83±0.08	0.66±0.04	0.74±0.09	0.75±0.01	0.79±0.07	0.59±0.05	0.59±0.04	0.69±0.03	0.70±0.02
MT1c	1.00±0.10	0.78±0.07	1.50±0.03	1.38±0.06	1.46±0.11	1.27±0.08	1.20±0.10	0.88±0.14	1.09±0.04	0.99±0.08
MT2a	1.00±0.09	0.90±0.07	1.03±0.08	0.96±0.05	0.96±0.05	0.84±0.04	1.15±0.07	2.47±0.28	1.01±0.08	2.16±0.27
MT2b	1.00±0.04	0.86±0.08	0.95±0.03	0.92±0.03	0.93±0.03	0.90±0.03	0.98±0.10	1.03±0.08	0.86±0.03	0.92±0.03
MT3	1.00±0.09	0.88±0.06	1.15±0.06	1.04±0.12	1.27±0.08	1.10±0.08	1.29±0.17	1.31±0.18	1.02±0.08	0.97±0.07

In roots as well as in leaves, no changes in H_2O_2 content were detected after Cd exposure in wild type or *mpk6* mutant plants (Figure 7.1.A and B). The amount of membrane damage, induced by the production of ROS after Cd exposure, was measured by the analysis of thiobarbituric acid reactive metabolites (TBArm). In roots, TBArm levels of non-exposed *mpk6* mutant plants were significantly lower than in non-exposed wild types (Figure 7.1.C). After exposure to Cd, wild type plants showed a slightly increasing trend in lipid peroxidation. In contrast, lipid peroxidation was reduced in Cd-exposed *mpk6* knockout plants after 24h, leading to a significant genotype*treatment interaction effect ($p < 0.0001$). In leaves, no significant differences in lipid peroxidation were observed after exposure to Cd (Figure 7.1.D).

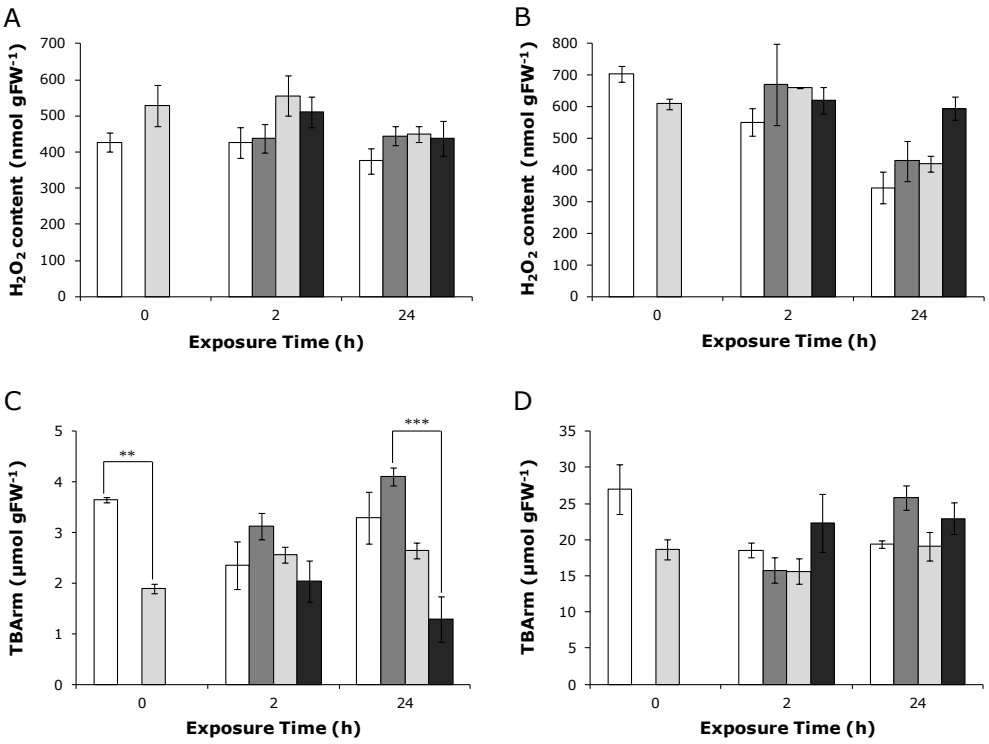

Figure 7.1.: *H_2O_2 content (nmol gFW^{-1}) and lipid peroxidation (µmol gFW^{-1}) in roots (**A**-**C**) and leaves (**B**-**D**) of 3-week-old Arabidopsis thaliana wild type and mpk6 knockout plants exposed to 5 µM $CdSO_4$ during 0, 2 and 24h (□ = wild type non-exposed, ■ = wild type exposed, □ = mpk6 non-exposed, ■ = mpk6 exposed). Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: treatment effect: ***: $p < 0.01$; **: $p < 0.05$).*

To measure the cellular redox state, the amount of reduced and oxidized glutathione was analyzed in Cd-exposed wild type and *mpk6* knockout seedlings (Table 7.3.). Roots of wild type plants showed significantly reduced levels of GSH and GSSG after 2h Cd exposure, resulting in reduced levels of total glutathione and a sustained GSSG/GSH ratio. After 24h exposure to Cd, GSH and GSSG concentrations were increased in wild type plants and similar to the levels in control plants. *Mpk6* mutant seedlings also showed after 2 and 24h Cd exposure a significant reduction in GSH and total glutathione levels in roots, but in contrast, GSSG content remained unaltered resulting in an increased GSSG/GSH ratio. In leaves, non-exposed *mpk6* mutant plants showed a significantly lower concentration of GSSG at the onset of the day leading to a reduced GSSG/GSH ratio as compared to wild type plants. After 24h exposure to Cd, the GSSG content and consequently the GSSG/GSH ratio in the leaves were significantly reduced in wild type plants. In leaves of *mpk6* mutants, concentrations of GSSG and the GSSG/GSH ratio were also decreased after 24h exposure to Cd, although not statistically significant.

Activities of enzymes involved in the antioxidative defence were measured in roots and leaves of wild type and *mpk6* knockout seedlings exposed to Cd (Table 7.4.). In roots, the activity of glutathione reductase (GR) and catalase (CAT) was induced in wild type seedlings after 24h Cd exposure, whereas in *mpk6* mutants only the CAT activity was increased. In leaves, the activity of the superoxide dismutase (SOD) was significantly elevated in *mpk6* mutants after 2h exposure to Cd whereas no changes in SOD activity were seen in wild type plants. After 24h exposure to Cd, the activity of GR was significantly reduced in both wild type and *mpk6* mutant seedlings.

Transcript levels of pro- and antioxidative enzymes were also measured in wild type and *mpk6* knockout seedlings after exposure to Cd. Concerning the pro-oxidative lipoxygenases and NADPH oxidases, gene expression of *RBOHD* was significantly increased in the roots of wild type plants, not in *mpk6* mutant plants, after 24h exposure to Cd (Table 7.5.). No significant differences were observed for the transcript levels of the other pro-oxidative genes. In the leaves, expression of *LOX2* and *RBOHD* was increased in response to Cd exposure in both wild type and *mpk6* mutant plants (Table 7.6.).

Table 7.3.: *Concentration (nmol gFW^{-1}) of reduced (GSH) and oxidized (GSSG) glutathione in roots and leaves of 3-week-old Arabidopsis thaliana wild type and mpk6 knockout plants exposed to 5 µM $CdSO_4$ during 0, 2 and 24h (GSSG levels were expressed in GSH equivalents). Values are mean ± S.E. of ± 4 biologically independent replicates (significance level: treatment effect: ***: p < 0.01; **: p < 0.05; genotype or genotype*treatment interaction effect: a: p < 0.01; b: p < 0.05).*

ROOTS					
		wild type		*mpk6*	
Time	Metabolite	Control	5 µM Cd	Control	5 µM Cd
0h	GSH + GSSG	150.5 ± 14.5		148.5 ± 2.4	
	GSH	147.1 ± 15.0		144.6 ± 1.7	
	GSSG	3.4 ± 1.1		3.9 ± 1.3	
	GSSG/GSH	0.024 ± 0.008		0.027 ± 0.009	
2h	GSH + GSSG	168.6 ± 11.4	55.9 ± 2.7***	150.4 ± 4.6	62.4 ± 3,8***
	GSH	149.7 ± 8.6	53.9 ± 2.5***	149.6 ± 4.8	60.2 ± 3.5***
	GSSG	18.9 ± 7.3	2.0 ± 0.5***	0.8 ± 0.2[a]	2.2 ± 0.4
	GSSG/GSH	0.163 ± 0.039	0.037 ± 0.008	0.006 ± 0,002[a]	0.035 ± 0,007***
24h	GSH + GSSG	148.7 ± 9.8	106.6 ± 9.9	150.8 ± 5.7	99.6 ± 8.1**
	GSH	134.7 ± 13.7	97.8 ± 9.5	148.2 ± 5.7	91.6 ± 6.5***
	GSSG	13.9 ± 6.5	8.8 ± 1.0	2.6 ± 0.6	8.1 ± 2.0
	GSSG/GSH	0.117 ± 0.058	0.092 ± 0.011	0.014 ± 0.002	0.086 ± 0.018**

LEAVES					
		wild type		*mpk6*	
Time	Metabolite	Control	5 µM Cd	Control	5 µM Cd
0h	GSH + GSSG	379.6 ± 58.2		341.3 ± 51.8	
	GSH	336.6 ± 58.4		316.8 ± 34.9	
	GSSG	43.0 ± 1.6		23.8 ± 3.8[a]	
	GSSG/GSH	0.138 ± 0.021		0.090 ± 0.018	
2h	GSH + GSSG	464.9 ± 41.8	358.4 ± 47.5	404.1 ± 29.5	391.5 ± 50.8
	GSH	391.9 ± 21.3	411.6 ± 87.8	365.6 ± 30.7	356.3 ± 57.6
	GSSG	50.7 ± 10.4	35.6 ± 10.0	47.2 ± 4.0	35.2 ± 9.9
	GSSG/GSH	0.134 ± 0.032	0.082 ± 0.020	0.129 ± 0.005	0.117 ± 0.043
24h	GSH + GSSG	277.3 ± 10.3	389.1 ± 9.5	329.0 ± 35.6	312.7 ± 26.7
	GSH	244.6 ± 25.3	360.0 ± 12.3	304.5 ± 35.9	290.3 ± 22.7
	GSSG	56.9 ± 0.3	28.1 ± 2.3**	24.4 ± 4.0[b]	11.8 ± 3.3
	GSSG/GSH	0.260 ± 0.014	0.082 ± 0.010***	0.084 ± 0.015[a]	0.039 ± 0.008

Table 7.4.: *Enzyme activities (U gFW^{-1} for SOD, mU gFW^{-1} for all other enzymes) in roots and leaves of 3-week-old Arabidopsis thaliana wild type and mpk6 knockout plants exposed to 5 µM $CdSO_4$ during 0, 2 and 24h. Values are mean ± S.E. of ± 5 biologically independent replicates (significance level: treatment effect: ***: p < 0.01; **: p < 0.05; genotype or genotype*treatment interaction effect: a: p < 0.01; b: p < 0.05).*

ROOTS					
		wild type		*mpk6*	
Time	Enzyme	Control	5 µM Cd	Control	5 µM Cd
0h	SOD	3069 ± 397		3435 ± 89	
	SPOD	7619 ± 710		6176 ± 619	
	GPOD	24625 ± 2608		24617 ± 277	
	GR	498 ± 49		592 ± 62	
	CAT	67 ± 5		58 ± 1	
2h	SOD	2726 ± 303	2602 ± 70	1807 ± 273^{b}	2669 ± 109
	SPOD	7177 ± 907	4958 ± 469	4661 ± 536	5841 ± 503
	GPOD	21925 ± 1175	23954 ± 1695	19785 ± 1320	20072 ± 1158
	GR	504 ± 25	695 ± 56	516 ± 54	525 ± 48
	CAT	65 ± 9	70 ± 3	58 ± 1	60 ± 1
24h	SOD	2083 ± 295	2700 ± 125	1667 ± 114	2103 ± 93
	SPOD	8061 ± 1160	11029 ± 414	7038 ± 961	5845 ± 1000^{a}
	GPOD	21205 ± 1669	21106 ± 1378	14839 ± 456	15549 ± 1023
	GR	530 ± 12	774 ± 26**	504 ± 24	597 ± 54
	CAT	69 ± 4	116 ± 4***	61 ± 6	79 ± 3^{**,a}

LEAVES					
		wild type		*mpk6*	
Time	Enzyme	Control	5 µM Cd	Control	5 µM Cd
0h	SOD	410 ± 55		701 ± 249	
	SPOD	2475 ± 31		2793 ± 60	
	GPOD	1413 ± 85		1306 ± 52	
	GR	960 ± 211		1567 ± 24	
	CAT	474 ± 18		704 ± 26	
2h	SOD	1012 ± 63	1261 ± 70	872 ± 83	1589 ± 50***
	SPOD	2988 ± 141	2559 ± 136	2708 ± 110	2645 ± 104
	GPOD	1384 ± 113	1318 ± 80	1242 ± 12	1422 ± 53
	GR	1486 ± 67	1419 ± 16	1463 ± 46	1330 ± 68
	CAT	799 ± 60	818 ± 67	905 ± 35	917 ± 31
24h	SOD	1151 ± 163	1138 ± 77	1197 ± 33	1537 ± 99
	SPOD	3388 ± 273	3602 ± 239	3167 ± 151	3512 ± 297
	GPOD	1315 ± 63	1587 ± 23	1470 ± 36	1780 ± 90
	GR	1348 ± 29	875 ± 59***	1440 ± 101	1019 ± 31***
	CAT	762 ± 25	786 ± 4	848 ± 6	853 ± 28

With regard to the antioxidative enzymes, transcript levels of *FSD1* were elevated after 24h exposure to Cd in roots of wild type and *mpk6* mutant plants (Table 7.5.). In the leaves, transcript levels of *CAT2* were significantly reduced in both wild type and *mpk6* knockout plants after 24h exposure to Cd (Table 7.6.). In addition, gene expression of *CSD2* was significantly downregulated in leaves of wild type plants after 24h Cd exposure, whereas *mpk6* mutant seedlings showed a decreasing trend in *CSD2* transcript levels.

Because gene expression of *CSD*s is known to be regulated by miRNA398, primary transcripts of *CSD1* (*hnCSD1*), *CSD2* (*hnCSD2*) and the different isoforms of *miRNA398* (*pri-MIR398a*, *pri-MIR398b*, *pri-MIR398c*) were measured. The roots of wild type as well as *mpk6* mutant seedlings showed no change in *hnCSD1* or *hnCSD2* transcript levels after Cd exposure (Table 7.5.). Gene expression of *pri-MIR398a*, *pri-MIR398b* and *pri-MIR398c* was significantly increased after 2 or 24h Cd exposure respectively in both wild type and *mpk6* knockout plants. In leaves, an overall increase in transcript levels of *pri-MIR398a*, *pri-MIR398b* and *pri-MIR398c* was observed after 24h exposure to Cd (Table 7.6.).

7.2.3. Transcript levels of the MAPKinase MPK3 and the transcription factors *WRKY22*, *WRKY25* and *ZAT12* are regulated by MPK6 after Cd exposure

Because MPK6 is a signalling molecule, able to phosphorylate transcription factors, gene expression of the transcription factors *WRKY22/25/29* and *ZAT12* was measured in roots and leaves of wild type and *mpk6* knockout plants exposed to Cd. Transcript levels of *MPK3*, a MAPK homolog of MPK6 sharing the same function, were also analyzed.

Roots (Table 7.5.) of wild type plants showed a significant increase in *MPK3* expression after 2 and 24h Cd exposure whereas no changes in *MPK3* transcript levels were seen in *mpk6* knockout plants. Gene expression of *WRKY22* was significantly reduced in roots of *mpk6* mutant plants after 24h exposure to Cd, while no changes were seen in wild type seedlings. In contrast, expression of *WRKY25* was elevated in roots of 24h Cd-exposed wild type plants, whereas no changes in gene expression were seen in the Cd-exposed *mpk6* mutants.

Table 7.5.: *Transcript levels in roots of 3-week-old wild type and mpk6 knockout Arabidopsis thaliana plants exposed to 5 µM $CdSO_4$ during 0, 2 or 24h. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulated: p<0.01 p < 0.001; upregulated: p<0.01 p < 0.001).*

ROOTS										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	**wild type**	***mpk6***	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**
LOX1	1.00±0.02	0.76±0.08	1.55±0.14	1.24±0.05	1.36±0.06	1.17±0.11	2.47±0.38	5.04±1.17	1.77±0.19	2.93±0.72
RBOHC	1.00±0.06	1.08±0.07	0.92±0.02	1.00±0.06	1.14±0.03	1.10±0.08	1.18±0.12	1.25±0.12	1.52±0.03	1.54±0.06
RBOHD	1.00±0.21	0.61±0.05	0.91±0.16	1.44±0.34	0.54±0.04	0.97±0.36	1.19±0.01	2.09±0.08	0.88±0.07	1.27±0.19
CSD1	1.00±0.09	0.89±0.11	1.01±0.05	1.04±0.04	0.95±0.04	1.01±0.04	0.96±0.06	0.78±0.14	0.91±0.06	0.66±0.10
CSD2	1.00±0.04	0.83±0.06	0.88±0.04	0.91±0.06	0.90±0.08	0.93±0.09	1.16±0.13	0.92±0.14	0.90±0.04	0.73±0.09
FSD1	1.00±0.29	1.38±0.31	2.70±0.77	1.90±0.04	2.38±0.45	0.89±0.44	2.42±0.70	22.3±7.32	2.88±0.25	16.0±5.06
APX1	1.00±0.05	0.92±0.09	1.05±0.05	1.14±0.02	1.12±0.05	1.14±0.02	1.01±0.10	0.87±0.04	1.02±0.02	0.80±0.05
CAT1	1.00±0.06	1.00±0.10	0.77±0.04	0.81±0.04	0.76±0.04	0.90±0.11	1.00±0.02	1.05±0.10	0.82±0.04	0.83±0.05
CAT2	1.00±0.05	1.22±0.08	1.07±0.19	0.85±0.04	1.12±0.08	1.02±0.05	1.40±0.08	1.01±0.15	1.42±0.11	1.31±0.12
CAT3	1.00±0.11	1.02±0.07	0.89±0.10	0.98±0.10	0.75±0.12	0.76±0.04	1.13±0.15	0.60±0.03	1.01±0.12	0.77±0.12
MPK3	1.00±0.07	0.98±0.08	0.95±0.08	1.86±0.37	1.05±0.11	0.89±0.08	1.27±0.07	2.32±0.06	1.40±0.06	1.71±0.15
WRKY22	1.00±0.23	1.42±0.32	1.21±0.14	1.12±0.20	1.10±0.09	0.87±0.14	1.48±0.21	0.70±0.08	2.24±0.35	0.78±0.03
WRKY25	1.00±0.13	1.32±0.17	1.26±0.14	1.05±0.20	1.48±0.14	1.67±0.20	1.23±0.09	2.71±0.19	1.74±0.15	2.26±0.38
WRKY29	1.00±0.18	1.61±0.16	1.35±0.12	1.83±0.28	1.64±0.22	2.21±0.48	1.38±0.20	1.89±0.15	1.69±0.14	1.77±0.23
ZAT12	1.00±0.20	0.78±0.06	0.16±0.05	2.21±0.90	0.43±0.13	0.65±0.37	0.48±0.10	6.29±0.63	0.67±0.03	3.43±1.44
hnCSD1	1.00±0.10	0.86±0.09	0.84±0.04	1.21±0.13	0.90±0.04	0.98±0.11	0.97±0.08	1.04±0.03	0.95±0.05	1.04±0.02
hnCSD2	1.00±0.07	0.73±0.07	0.80±0.07	0.94±0.11	0.85±0.05	0.76±0.05	1.12±0.05	1.05±0.12	0.77±0.04	0.93±0.05
pri-MIR398a	1.00±0.03	0.93±0.10	0.84±0.03	6.99±0.97	0.92±0.13	9.35±0.36	1.03±0.11	1.40±0.20	0.91±0.10	1.26±0.15
pri-MIR398b	1.00±0.14	0.82±0.11	0.92±0.08	0.86±0.09	0.88±0.11	0.69±0.04	1.08±0.22	7.04±1.90	1.00±0.15	8.13±2.15
pri-MIR398c	1.00±0.12	0.81±0.08	0.90±0.06	0.93±0.12	0.93±0.10	0.68±0.09	0.99±0.15	7.70±2.10	0.88±0.16	9.51±2.84

Table 7.6.: *Transcript levels in leaves of 3-week-old wild type and mpk6 knockout Arabidopsis thaliana plants exposed to 5 µM $CdSO_4$ during 0, 2 or 24h. Gene expression was calculated relative to the non-exposed wild type plants (=1). Values are mean ± S.E. of at least 4 biologically independent replicates (significance levels of changes relative to the non-exposed genotype: downregulation:* p<0.01 | p < 0.001 *; upregulation:* p<0.01 | p < 0.001 *).*

LEAVES										
	0h		2h				24h			
			wild type		*mpk6*		wild type		*mpk6*	
Gene	**wild type**	***mpk6***	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**	**Control**	**5 µM Cd**
LOX2	1.00±0.13	0.81±0.05	4.87±0.28	5.94±0.50	4.80±0.57	4.28±0.29	1.06±0.06	1.78±0.09	1.26±0.07	2.04±0.12
RBOHC	1.00±0.03	1.04±0.06	1.02±0.03	1.18±0.05	1.07±0.08	1.05±0.04	1.05±0.06	1.78±0.23	1.11±0.03	2.16±0.46
CSD1	1.00±0.17	0.94±0.08	0.80±0.18	1.05±0.25	0.76±0.08	1.04±0.15	1.12±0.18	0.52±0.13	1.02±0.06	0.65±0.13
CSD2	1.00±0.28	1.06±0.13	0.55±0.07	0.86±0.23	0.87±0.20	1.12±0.19	1.35±0.01	0.54±0.20	0.99±0.20	0.73±0.16
FSD1	1.00±0.50	0.17±0.04	1.50±0.60	1.17±0.56	1.96±0.54	0.96±0.55	0.18±0.02	1.05±0.41	1.18±0.57	1.34±0.67
APX1	1.00±0.05	1.05±0.09	1.66±0.16	1.79±0.02	2.24±0.13	1.74±0.15	1.16±0.07	1.29±0.15	1.43±0.09	1.39±0.06
CAT1	1.00±0.06	0.90±0.09	0.80±0.11	0.97±0.10	1.19±0.11	0.95±0.13	0.88±0.08	1.05±0.14	0.94±0.05	1.24±0.10
CAT2	1.00±0.03	1.04±0.11	0.67±0.06	0.75±0.09	0.84±0.07	0.73±0.02	1.13±0.09	0.88±0.01	1.29±0.03	0.77±0.04
CAT3	1.00±0.04	1.09±0.11	1.78±0.23	1.76±0.14	2.39±0.28	2.32±0.32	1.11±0.10	1.51±0.22	1.36±0.08	1.28±0.11
MPK3	1.00±0.02	1.07±0.12	1.27±0.07	1.76±0.08	1.70±0.26	1.47±0.08	1.39±0.17	4.85±0.80	1.39±0.14	3.78±0.45
WRKY22	1.00±0.03	1.06±0.10	0.58±0.05	0.63±0.03	0.77±0.06	0.85±0.07	0.97±0.07	0.50±0.01	1.04±0.06	1.04±0.14
WRKY25	1.00±0.05	1.14±0.12	1.51±0.32	2.01±0.16	2.31±0.39	1.26±0.04	1.69±0.26	17.1±5.09	1.55±0.19	12.8±2.28
WRKY29	1.00±0.08	1.07±0.07	1.29±0.10	1.49±0.18	2.52±0.15	2.80±0.09	1.05±0.12	0.71±0.09	1.48±0.14	1.39±0.08
ZAT12	1.00±0.10	1.74±0.20	0.36±0.10	1.28±0.74	0.64±0.46	0.30±0.04	0.32±0.04	6.70±1.70	0.55±0.04	4.88±0.86
hnCSD1	1.00±0.01	1.22±0.09	1.34±0.05	1.58±0.09	1.41±0.09	1.22±0.05	1.16±0.03	1.49±0.07	1.28±0.05	1.20±0.01
hnCSD2	1.00±0.03	0.88±0.11	0.53±0.01	0.60±0.04	0.53±0.03	0.54±0.04	0.60±0.08	0.39±0.03	0.67±0.04	0.44±0.07
pri-MIR398a	1.00±0.08	0.64±0.05	0.96±0.15	0.83±0.03	0.95±0.07	0.92±0.09	1.02±0.03	4.90±0.97	1.05±0.18	10.2±1.64
pri-MIR398b	1.00±0.34	0.85±0.39	2.06±0.67	0.85±0.11	2.64±0.53	1.60±0.82	2.00±0.71	9.56±2.54	3.11±1.10	6.32±2.04
pri-MIR398c	1.00±0.31	0.42±0.12	2.74±1.01	1.05±0.11	3.82±0.27	1.78±0.95	1.29±0.32	11.3±3.21	2.26±0.43	8.43±1.08

Transcript levels of *ZAT12* were elevated in roots of wild type plants after 2h Cd exposure, which continued after 24h. A delayed increase in gene expression of *ZAT12* was observed in *mpk6* knockout seedlings only after 24h exposure to Cd.
In the leaves (Table 7.6.), transcript levels of the transcription factor *WRKY22* were significantly reduced in wild type plants after 24h Cd exposure. In contrast, no changes in *WRKY22* expression were seen in Cd-exposed *mpk6* mutant plants. Expression of the transcription factor *ZAT12* was higher in non-exposed *mpk6* mutant seedlings than in non-exposed wild type plants. However, *ZAT12* transcript levels were elevated in both wild type and *mpk6* knockout plants after 24h Cd exposure. In addition, gene expression of *MPK3* was upregulated in both genotypes after 24h exposure to Cd.

7.3. Discussion

In earlier studies, it was shown that exposure of *Arabidopsis thaliana* seedlings to environmental realistic Cd concentrations induces oxidative stress (Smeets *et al*. 2009, Cuypers *et al*. 2011a) and that induction of MAPK gene expression suggested a role for MAPK cascades in the Cd stress response (Chapter 4; Opdenakker *et al*. 2012a; chapter 4). In this chapter, the role of MPK6 in Cd-induced oxidative stress was investigated after exposure of 3-week-old wild type and *mpk6* knockout *Arabidopsis thaliana* seedlings to 5 µM Cd during 0, 2 and 24h, via the evaluation of oxidative stress related parameters.
Although roots of *mpk6* mutant plants seem to take up less Cd, Cd accumulation is not statistically different after 24h Cd exposure (Table 7.1.). Once inside the cell, Cd ions are bound and sequestrated by metal chelators, like PCs and MTs, to prevent damage induced by free Cd ions (Hall 2002, Clemens 2006). Whereas no immediate changes in transcript levels of *PCS1* and most *MTs* were noticed, *MT2a* transcript levels elevated after 2h Cd exposure in *mpk6* knockout plants (Table 7.2.). MT2a is described to function as a metal chaperone for the delivery of essential metals to organelles and metal-requiring proteins (Guo *et al*. 2003, 2008) indicating that nutrient balance, although total amounts were similar, might be affected.
Glutathione also functions as a metal chelator in plant cells. It can directly bind and sequester free Cd ions in the cytosol or subcellular compartments or it can be used in the synthesis of PCs. The sharp decrease in total GSH levels after 2h

of exposure suggests that in Cd-exposed roots, GSH is immediately being consumed for the binding and sequestration of the free Cd or in the synthesis of PCs (Jozefczak, personal communication). Besides its function in metal chelation, GSH also acts in ROS scavenging by neutralizing ROS via donation of an electron from its thiol group. By doing so, GSH itself becomes oxidized. GSSG can be reduced back to GSH by the enzyme glutathione reductase (GR), which uses NADPH to reduce GSSG (reviewed in Jozefczak *et al*. 2012). In wild type plants, after 2h Cd exposure, GSH is mainly used for chelation purposes as also GSSG levels dropped and the cellular redox state was maintained. The drop in total and reduced GSH was also observed at 2h Cd exposure in *mpk6* mutants, also pointing towards a direct involvement of GSH in this mutant. Strikingly, GSSG levels in the *mpk6* mutant were lower already in control conditions at this time point, leading to similar levels of GSSG after 5 µM Cd exposure and hence an increased cellular redox state. After 24h the GSH levels recovered, possibly due to *de novo* synthesis, but in the mutants they were still significantly lower than in wild type plants (Table 7.3.). The increased levels of GR activity in wild-type plants after Cd exposure also led to the maintenance of GSSG levels, whereas the unaltered GR activity in the *mpk6* mutant combined with the 5 µM Cd-induced stress led to increased GSSG levels. So in both genotypes at 24h Cd exposure, GSH is necessary for both antioxidative defence and metal chelation, but the *mpk6* mutants seems to recover more slowly from the decrease in GSH seen after 2h. Temporary or sustained depletion of GSH can influence the antioxidative capacity and consequently cause oxidative stress (Seth *et al*. 2012).

Regarding the above-mentioned decrease in GSH, both genotypes might experience oxidative stress after Cd exposure. However, no changes in H_2O_2 levels were detected in roots of both wild type and *mpk6* knockout plants after Cd exposure (Figure 7.1.A). This can be due to the increase in catalase activity after 24h exposure to Cd (Table 7.4.), which neutralizes H_2O_2 in the cell (Mhamdi *et al*. 2010). In accordance with this absence of increased H_2O_2 levels, lipid peroxidation was also not altered in roots of Cd-exposed wild type plants (Figure 7.1.C) and even reduced after Cd exposure in *mpk6* mutant seedlings. This indicates that both genotypes can cope with the Cd-imposed stress, but

based on our results, the role of MPK6 in the regulation of the cellular redox status in roots of *Arabidopsis* plants requires further investigation.

In contrast to Cd-induced ROS production and oxidative injury in roots of *Arabidopsis* plants, which is a secondary effect not detected before 24h Cd exposure (Opdenakker *et al*. 2012a: chapter 4, Cuypers *et al*. 2011a), signalling pathways leading to changes in pro- and anti-oxidative gene expression may be triggered earlier. Therefore, the involvement of MPK6 in Cd-induced signalling pathways was investigated. Our data suggest that MPK6 positively regulates transcript levels of the closely related MAPKinase *MPK3* and the transcription factors *WRKY25* and *ZAT12* in *Arabidopsis* roots after exposure to Cd (Table 7.5.). The MPK6 mediated upregulations have not been reported before under Cd exposure. WRKY25 is known to regulate the expression of *APX1* and *APX2* in heat stress (Li *et al*. 2009). ZAT12 is involved in the transcriptional control of pro- and antioxidative enzymes, like *APX1*, *CSD1*, *CSD2*, *FSD1*, *RBOHD and LOX4* (Rhizhsky *et al*. 2004, Davletova *et al*. 2005a,b, Vogel *et al*. 2005). Indeed, gene expression of *RBOHD* in the roots was induced in wild type plants, but not in *mpk6* mutants (Table 7.5.). Based on the fact that (1) *ZAT12* transcript levels in the roots were increased by MPK6 after 2h Cd exposure and (2) ZAT12 is reported to control gene regulation of *RBOHD* under oxidative stress conditions, we hypothesize that MPK6-mediated gene expression of *RBOHD* in Cd-exposed roots occurs via ZAT12. However, a delayed upregulation of the *ZAT12* transcript levels was also observed in *mpk6* knockouts after 24h Cd exposure, indicating that ZAT12 can also be regulated via a MPK6-independent pathway in the absence of MPK6. Possibly, the closely related homolog of MPK6, MPK3, can be responsible for this bypass since transcript levels of *MPK3* were increased by MPK6 after Cd exposure.

In the leaves, Cd accumulated to similar levels in both wild type and *mpk6* mutants after exposure to Cd (Table 7.1.), indicating that MPK6 has no effect on the translocation of Cd from root to shoot in *Arabidopsis* plants. Whether Cd ions transported to the leaves were complexed with PCs upon arrival, was investigated in Cd-exposed wild type and *mpk6* knockout plants at the transcript level. *PCS1* expression was upregulated similarly in both genotypes. In contrast to the roots where a sharp decline was observed after Cd exposure, no

alterations in GSH concentrations in leaves were observed. Phytochelatin production in leaves was previously observed in *Arabidopsis* plants exposed during 1 week to 1 or 10 µM Cd (Semane *et al*. 2007). Although the total amount of GSH diminished slightly, GSH biosynthesis genes were induced. In our 24h experimental set-up, GSH biosynthesis is possibly maintained and in addition can provide substrates for PC synthesis. Upon Cd exposure, Mn levels decreased significantly, and a lower leaf Cu content was observed in *mpk6* knockout seedlings (Table 7.1). Manganese is an essential micronutrient for plants which can function as a cofactor in enzymes, like in Mn-containing superoxide dismutase protecting cells from the damaging effects of ROS (Hänsch and Mendel 2009).

Whereas GSH has a crucial role in Cd chelation and sequestration (Seth *et al*. 2012, Jozefczak *et al.* 2012), it makes up an essential part of the cellular redox state. Interestingly, the GSSG level was lower under control conditions in *mpk6* mutants as compared to wild type plants, which disappeared during the day (Table 7.3.). In agreement with observations in the roots, further investigation is required to unravel the role of MPK6 in the GSH redox state in combination with a circadian rhythm. Nevertheless, after Cd exposure, the GSH metabolism was similarly affected in both genotypes (Tables 7.3 and 7.4.). It was previously shown that the GSH metabolism is crucial in the regulation of antioxidative enzymes during Cd stress (Cuypers *et al*. 2011a). As MPK6 affects the GSSG levels, the role of MPK6 in the control of the cellular redox status in Cd-exposed leaves as well as ROS production and possible oxidative damage were investigated. Neither increased H_2O_2 levels, nor lipid peroxidation were observed in leaves of both genotypes under Cd exposure (Figure 7.1.). The absence of H_2O_2 production and lipid peroxidation in *Arabidopsis* leaves after short term Cd exposure was earlier reported by Opdenakker *et al*. (2012a; chapter 4). They postulated that the low Cd levels in the leaves initially do not cause cellular damage but rather induce signalling responses to cope with the existing stress. Therefore, transcript levels of signalling components as well as pro- and antioxidative enzymes were analyzed and compared between wild type and *mpk6* knockout seedlings to identify MPK6 dependent regulation under Cd stress. Although expression of the transcription factors *WRKY25* and *ZAT12* as well as of downstream targets (*RBOHD*, *LOX2*, *CAT2*) was increased in Cd-

exposed leaves, their gene regulation seems to be not mediated by MPK6 as was observed in the roots. This indicates that transcript levels of *WRKY25*, *ZAT12* and downstream signalling targets in leaves exposed to Cd can be regulated by other MPK6-independent signalling pathways. However, gene expression of *WRKY22* was demonstrated to be under the control of MPK6 in leaves of Cd-exposed *Arabidopsis* plants (Table 7.6.). Asai *et al*. (2002) demonstrated earlier that WRKY22 is activated by MPK6 in flagellin signalling in *Arabidopsis*. Concerning the downstream targets of these signalling pathways, transcript levels of *CSD2* were decreased in leaves of Cd-exposed wild type plants (Table 7.6.), whereas no changes were detected in *mpk6* knockouts, suggesting that MPK6 is involved in controlling the gene expression of *CSD2*.

Gene expression of *CSD1/2* is regulated by miRNA398. Under non-stressed conditions, *miRNA398* is expressed at moderate levels keeping *CSD1/2* expression at normal levels. However, *miRNA398* is downregulated under oxidative stress and upregulated under Cu deprivation leading to respectively high or low transcript levels of *CSD1/2* (Sunkar *et al*. 2012). Cuypers *et al*. (2011a) demonstrated that exposure of *Arabidopsis* seedlings to environmentally realistic Cd concentrations (5 µM $CdSO_4$) during 24h increased levels of mature miRNA398b/c, which in turn led to reduced *CSD1* and *CSD2* transcript levels. It was found here that the upregulation of the *pri-MIR398b* transcripts was less strong in *mpk6* mutants than in wild types, and this may correlate with a lack of decrease in *CSD2* mRNA levels in the mutant, suggesting a role for MPK6 in the miRNA398-mediated downregulation of CSD2. MPK6 was earlier reported to be involved in gene regulation of CSDs via miRNA398 in roots and leaves of *Arabidopsis* after exposure to Cu (chapter 6).

In conclusion, it was demonstrated that GSSG levels were lower in roots and leaves of non-exposed *mpk6* mutants in comparison to wild type plants and they were regulated by the circadian rhythm. However, after Cd exposure the same response in GSH and GSSG levels was observed in both wild type and *mpk6* mutants, indicating a role for GSH in metal chelation as well as in the antioxidative defence. Nevertheless, *mpk6* knockouts recovered more slowly with regard to GSH levels after Cd exposure. So, the role of MPK6 in the GSH metabolism clearly deserves further attention. In addition, after studying the

cellular redox status at the biochemical level, no clear role for MPK6 in the Cd-induced oxidative stress response in roots and leaves was observed. This can be explained by the fact that Cd is not a redox-active metal and stimulates ROS production via indirect mechanisms leading to a delayed signalling response seen after 24h. Consequently, oxidative stress and associated cellular damage are only initiated after 24h Cd exposure but may be more pronounced after longer exposure times (Opdenakker *et al*. 2012a; chapter 4). Therefore chronic Cd exposure experiments are needed to unravel the role of MPK6 in Cd-induced responses. Concerning the signalling responses after Cd exposure, clear differences between roots and leaves were observed. In the roots, Cd-induced MPK6 regulated transcript levels of the transcription factors WRKY25 and ZAT12. A possible target of ZAT12, the NADPH oxidase RBOHD, seemed also to be regulated by MPK6 at the transcriptional level after Cd exposure, resulting in an increased ROS production in Cd-exposed *Arabidopsis* roots. In leaves, we observed that MPK6 in Cd-exposed leaves is responsible for the transcriptional control of WRKY22 and that MPK6 may be involved in miRNA398b/c-mediated downregulation of *CSD2* mRNA levels, which indeed could be at the origin of oxidative stress beyond 24h Cd exposure.

Chapter 8

General discussion, conclusion and perspectives

8.1. Introduction

Metals, like cadmium (Cd) and copper (Cu), are toxic for organisms when they are present in the environment in too high concentrations. In plants, exposure to Cd and Cu results in growth retardation and disrupts physiological processes, such as photosynthesis and respiration. At the cellular level, Cd and Cu disturb the cellular redox balance by favouring the production of reactive oxygen species (ROS). On the one hand, ROS are harmful to the cell by damaging cellular components, like DNA, proteins and lipids. On the other hand, ROS can also act as signalling molecules mediating many normal physiological processes as well as defence responses against stress. Reactive oxygen species signalling in plants involves mitogen-activated protein kinase (MAPK) cascades, which regulate transcript levels of target genes via phosphorylation of transcription factors. Signalling via MAPKs is also associated with Cd and Cu stress.

Since knowledge about stress signalling at the cellular level is necessary to understand plant responses to metals, the main objective of this study was to investigate the role of MAPK cascades in metal stress signalling and in the establishment of cellular responses to these metals. Therefore, two metals (Cd and Cu) with distinct physico-chemical characteristics were applied to the plants. Copper is an essential element necessary for normal plant function, while Cd is non-essential and is already toxic to plants in low concentrations. In addition, Cu is redox-active, inducing ROS production directly because of its chemical properties whereas the non-redox-active Cd causes elevated ROS levels via indirect mechanisms. In the first part of this work (chapter 4), time course measurements were undertaken shortly after the Cd or Cu exposure to gain insight in the metal-dependent ROS production in roots and leaves of *Arabidopsis* plants. Via the transcriptional analysis of ROS-inducible MAPK cascade components in these Cd- and Cu-exposed plants, possible metal-induced signalling candidates were identified. In the following parts, the specific role of these MAPK signalling candidates, more specifically OXI1 (chapter 5) and MPK6 (chapter 6 and 7), was examined in Cd- or Cu-induced oxidative stress using *oxi1* and *mpk6* knockout plants.

8.2. Translocation or complexation of metals is mediated by OXI1 and MPK6 in Cu- or Cd- exposed *Arabidopsis thaliana*

Exposure to Cu or Cd is toxic to *Arabidopsis* plants, even when environmental realistic concentrations were applied to the plants. This was reflected at the morphological and biochemical level in alterations of growth parameters, like the reduction in root weight after Cu exposure, or the oxidative stress induced in Cu- and Cd-exposed roots and leaves.

A first line of defence against the toxic effects of Cu and Cd in the cell, is the complexation and sequestration of the metal ions by phytochelatins (PCs) and metallothioneins (MTs)(Clemens 2006, Verbruggen et al. 2009). Signalling components, like the protein kinase OXI1 and the MAPKinase MPK6, were observed to have an influence on this complexation and sequestrations of metals. However, this was insufficient to explain the increased tolerance for Cu excess of both *oxi1* and *mpk6* knockouts as compared to wild type plants. OXI1 seems to be responsible for Cu homeostasis in roots of wild type plants, possibly due to a better root hair development that is previously described to be OXI1-mediated (Anthony *et al*. 2004, Rentel *et al*. 2004)(chapter 5). In *mpk6* knockout plants, similar Cu concentrations were observed in the roots, but a significant accumulation of Cu was detected in leaves of *mpk6* knockout plants after exposure to Cu (chapter 6). Retention of Cu in the roots of wild type plants protects the leaves from the damaging effects of Cu but renders the roots more sensitive to Cu-induced oxidative stress. However, the role of MPK6 in Cu stress responses requires further attention as also elevated oxidative stress was observed in *mpk6* knockout roots and wild type leaves. In case of Cd exposure, MPK6 has no clear effect on the translocation of Cd from the root to the shoot but Cu translocation was diminished in Cd-exposed wild type plants. Complexation of Cd with glutathione (GSH) or PCs in Cd-exposed roots may occur immediately, based on the sharp decline of overall GSH contents (chapter 7). Recently, a link between protein phosphorylation by MAPKs and the synthesis of PCs was suggested after Cd exposure (Lima et al. 2012). However, the use of GSH for the sequestration of Cd induced a temporary depletion of GSH, which is also used as a scavenger of ROS leading to an induction of oxidative stress.

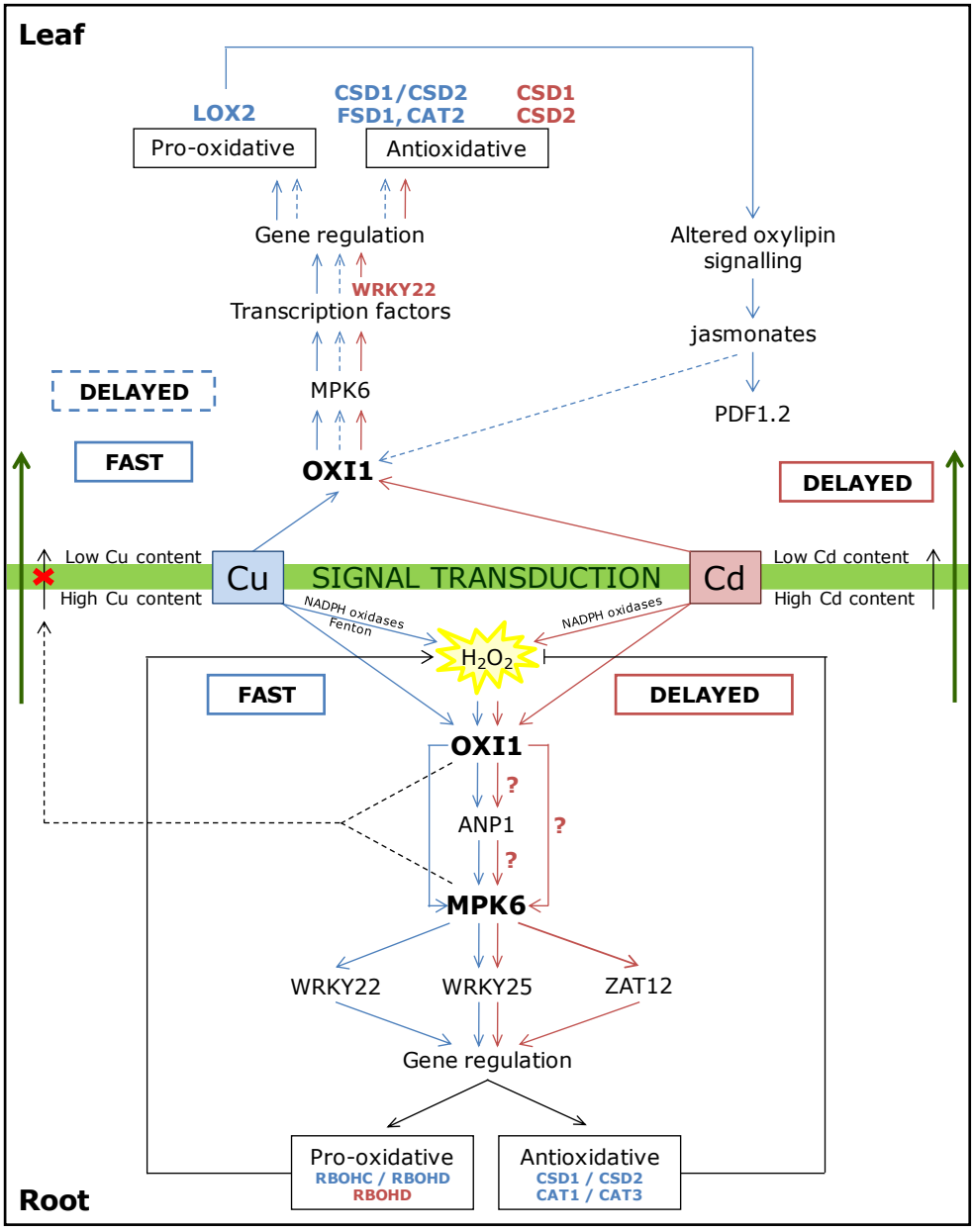

Figure 8.1.: *Overview of oxidative signalling pathways in Cu- or Cd-exposed Arabidopsis leaves and roots. Blue lines represent the fast (filled lines) and delayed (dashed lines) Cu-specific signalling pathways whereas red lines indicate Cd-dependent signalling cascades. Green arrows represent root-to-shoot signal transduction.*

8.3. The role of MAPK signalling in Cu- and Cd-induced oxidative stress in roots of *Arabidopsis thaliana*

At the cellular level, Cu exposure of the roots resulted in an elevated H_2O_2 concentration. Cu-mediated production of H_2O_2 can occur directly by Fenton reactions or indirectly by the activity of the NADPH oxidases RBOHC and RBOHD, whose transcript levels were rapidly elevated after Cu exposure (chapter 4). The NADPH oxidase-dependent production of $O_2^{\bullet-}$ and H_2O_2 can be used by Fenton and Haberweiss reactions for the formation of the more reactive $^{\bullet}OH$, which initiates lipid peroxidation directly after Cu exposure resulting in membrane damage. Lipid peroxidation can also be initiated by the action of lipoxygenases, enzymes, which catalyze the oxygenation of polyunsaturated fatty acids into lipid hydroperoxides (chapter 4). These lipid hydroperoxides can be further metabolized by different pathways resulting in the formation of oxylipins (Andreou *et al*. 2009), which also show signalling functions (Mithöfer *et al*. 2004). Exposure of roots to Cd caused a delayed ROS production supporting the idea that oxidative injury by Cd is a secondary effect. An induction of NADPH oxidases (RBOHD) was only observed after 24h. Although Cd exposure stimulated gene expression of lipoxygenases, no lipid peroxidation was detected suggesting that the lipoxygenases cause alterations in oxylipin signalling (chapter 4) (Figure 8.1.).

Besides its role as harmfull ROS initiating cellular damage, H_2O_2 can also act as a signalling molecule inducing MAPK cascades which regulate transcription of enzymes involved in pro- and antioxidative processes. After Cu exposure, a fast induction of the MAPK signalling pathway was observed (already 2h after Cu exposure), whereas Cd exposure showed a delayed induction of MAPK components after 24h (chapter 4). OXI1 was described in different studies as a ROS sensor, a protein kinase that is activated by ROS, and relays stress signals to the nucleus via activation of MAPKinases, such as MPK3 and MPK6 (Rentel *et al*. 2004, Petersen *et al*. 2009). After Cu exposure, a fast induction of *OXI1* transcript levels occurred pointing to a central role for OXI1 in Cu-mediated stress responses (chapter 4). Therefore, the signalling function of OXI1 under Cu stress was investigated by the use of *oxi1* knockout plants (chapter 5). OXI1 was found to regulate gene expression of ANP1, a MAPKKK that is also involved

in the activation of MPK3 and MPK6 (Kovtun *et al*. 2000). A role for OXI1 in the regulation of transcript levels of MPK3/6 was not detected, possibly because ANP1 is bypassing this function of OXI1. Consequently, further downstream responses of the signalling cascade were examined by using *mpk6* knockout seedlings (chapter 6 and 7). In Cu-exposed as well as in Cd-exposed roots, MPK6 regulated the transcription of its MAPK homolog MPK3 meaning that both MPK6 and MPK3 are needed for full stress signalling after metal exposure. After Cu exposure, MPK6 induced gene expression of the transcription factors WRKY22 and WRKY25, whereas Cd-dependent activation of MPK6 resulted in enhanced transcript levels of WRKY25 and ZAT12. This indicates that different metals can induce the same MAPK signalling pathway, which consequently activates different transcription factors initiating metal-dependent responses. Via these transcription factors, MPK6 resulted in the Cu-dependent transcription of the NADPH oxidases *RBOHC/D* and the antioxidative enzymes *CSD1/2* and *CAT1/3*. In addition, MPK6-induced *CSD1* gene expression in Cu-exposed roots was observed to be regulated via MPK6-mediated downregulation of miRNA398 (Figure 8.1.).

After exposure to Cd, only gene expression of *RBOHD* was regulated by MPK6. Induction of *RBOHC* and *RBOHD* stimulates the pro-oxidative side of the redox balance, leading to more ROS production, whereas the transcriptional regulation of the antioxidative enzymes results in removal of the potential damaging ROS from the cell (Figure 8.1.).

8.4. The role of MAPK signalling in Cu- and Cd-induced oxidative stress in leaves of *Arabidopsis thaliana*

Because of the retention of Cu in the roots of wild type plants, no elevation in Cu content of the leaves was seen. Consequently, an increase in ROS production and lipid peroxidation is possibly observed after more prolonged exposure to Cu. In case of Cd exposure, only low amounts of the Cd taken up by the roots were transported to the leaves. However, in comparison to leaf concentrations of essential micronutrients, like Cu and Zn, the Cd content in the leaves is relatively high. Although Cu and Cd levels of the leaves are lower as observed in the roots, leaf transcript levels of the OXI1 signalling cascade were rapidly induced after Cu exposure and only after 24h in Cd-exposed plants (chapter 4),

indicating the existence of root-to-shoot signalling (Remans *et al*. 2010, Cuypers *et al*. 2011a). Although transcript levels of pro- and antioxidative enzymes are regulated by Cu exposure in leaves, no specific involvement of OXI1 and MPK6 in this regulation were identified except for LOX2 (chapter 5 and 6). Jasmonate, an oxylipin that is synthesized starting from LOX2 activity, is known to play a role in Cu stress responses (Xiang and Oliver 1998, Maksymiec and Krupa 2006, Maksymiec *et al*. 2007). The plant defensin PDF1.2, a target of jasmonate signalling, was observed to be regulated by OXI1 in Cu-exposed leaves, indicating that OXI1 initiates oxylipin signalling in leaves of *Arabidopsis* plants exposed to Cu (chapter 5). The altered oxylipin signalling in Cu-exposed leaves can be the trigger for a second, delayed stimulation of the OXI1 signalling cascade resulting in changes of pro- and antioxidative genes. Indeed, leaves exposed to Cu for a longer time (1 week) displayed a MPK6-mediated regulation of *LOX2* and the antioxidative enzymes *CSD1, CSD2, FSD1* and *CAT2*. Like already mentioned for Cu-exposed roots, MPK6 was involved in the downregulation of *pri-MIR398* in leaves exposed to Cu during 1 week, allowing *CSD1* and *CSD2* transcript levels to increase after Cu exposure (Figure 8.1.).

In Cd-exposed leaves, a delayed activation of MAPK signalling pathways was also observed, preferentially stimulating the pro-oxidative side of the redox balance (RBOHC/D, LOX2). Consequently, LOX2 can interfere with the oxylipin signalling in leaves after Cd exposure. Involvement of MPK6 in this MAPK signalling cascade was demonstrated by the regulation of *WRKY22*, *CSD1* and *CSD2*, whose transcript levels were downregulated in leaves of Cd-exposed wild type plants whereas no changes were seen in *mpk6* knockouts after Cd exposure. The MPK6-mediated downregulation of *CSD1/2* after Cd exposure did not involve miRNA398, since transcript levels of *pri-MIR398* were elevated after Cd exposure in both wild type and *mpk6* mutant seedlings. However, it is possible that the MPK6-dependent reduction of *WRKY22* plays a role in the MPK6 mediated decrease of *CSD1/2* expression levels (Figure 8.1.).

8.5. Conclusion and future perspectives

In conclusion, MAPK signalling is important in the establishment of correct stress responses after metal exposure. In case of Cu exposure, a fast induction of these signalling cascades regulating pro- and antioxidative processes in the

roots was observed whereas a delayed induction was detected after Cd exposure. In contrast to the fact that excess Cu uptake is restricted to the roots and only relatively small amounts of Cd are transported to the leaves, MAPK signalling cascades are activated in the leaves after metal exposure indicating root-to-shoot signalling. Although metals with different physico-chemical characteristics (redox-active versus non-redox-active, essential versus non-essential) are able to induce the same MAPK cascades, the outcomes of these signalling pathways are metal-dependent by the use of metal-specific transcription factors. This was also demonstrated by the fact that knockouts of *OXI1* and *MPK6* were more tolerant to Cu than wild type plants while this tolerance was not detected after exposure to Cd. The Cu tolerance of *oxi1* mutants seemed to be related to an altered Cu uptake, whereas this needs further investigation in *mpk6* mutants (Figure 8.2.).

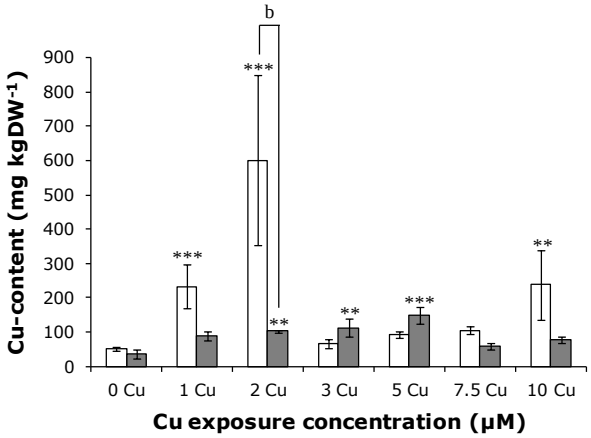

Figure 8.2.: *Cu content (mg kgDW^{-1}) of leaves of 2-week-old Arabidopsis thaliana wild type and oxi1 knockout seedlings exposed to different Cu concentrations during 1 week (white bars = wild type plants, grey bars = oxi1 mutant plants). Values are mean ± S.E. of at least 4 biologically independent replicates (significance level: treatment effect: **: $p < 0.05$, ***: $p < 0.001$; genotype* treatment interaction effect: b: $p < 0.05$).*

For future research, it is interesting to investigate the specific mechanisms responsible for the translocation of Cu from the root to the shoot in *oxi1* mutants. Indeed, a faster root-to-shoot translocation and the storage of the sequestered metals in the leaves are two of the three characteristics distinguishing hyperaccumulators from non-hyperaccumulating plants. Hyperaccumulation of metals is mostly caused by a different regulation and expression of genes encoding transmembrane transporters found in both kinds of plants (Rascio and Navari-Izzo 2011). Based on the results of this work, a role

for OXI1 in the regulation of these metal-accumulating processes can be postulated.

Metal uptake and distribution to different plant parts can also be influenced by the plant ecotype. In *Arabidopsis thaliana*, Landsberg erecta and Wassilewskija accessions are more tolerant to Cu exposure than the Columbia ecotype, although they are accumulating more Cu in their roots and leaves (Schiavon *et al*. 2007). This can be explained by a different tissue-specific partitioning of elements in the shoot between different ecotypes (Waters and Grusak 2008). In addition, Murphy and Taiz (1995) demonstrated that differences in Cu tolerance between distinct *Arabidopsis* ecotypes was closely correlated with alterations in the expression level of a 2-type metallothionein (MT2) gene.

Understanding plant processes controlling the uptake/exclusion, partitioning, root-to-shoot transfer, storage and detoxification of elements as well as knowledge about stress tolerance mechanisms is essential for safe and healthy food production or in contrast, the cleanup of metal-contaminated soils. Plants accumulating high concentrations of metals in their aerial, harvestable parts are of particular interest for phytoextraction of metal-contaminated soils, leading to long-term cleanup of polluted soils (Bhargava *et al*. 2012).

In humans, dietary intake of non-essential metals causes serious chronic health effects. To improve food safety, it is important to search for food crops with a reduced capacity to accumulate metals in edible parts. In contrast, to cope with dietary deficiencies of essential elements, selection for crop cultivars that absorb sufficient amounts of these elements from the soil and accumulate these in edible plant parts (biofortification) can improve human health. In addition, increasing the plant content of substances stimulating the absorption of essential mineral elements by the gut or reducing the amount of antinutrients (oxalate, polyphenolics, phytate) which interfere with their absorption, can help to improve human dietary uptake of essential elements. Examples of this kind of substances are ascorbic acid, beta-carotene and cysteine-rich polypeptides, which also posses antioxidative features (Mench *et al*. 2009, Singh *et al*. 2011).

Since polluted soils are most of the time contaminated with more than one metal, it is also interesting to include other metals, like Zn for instance, in this research and to investigate the role of multipollution.

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