PhD thesis presented on the 26th of November 2014 at Hasselt University

Members of the Jury

Prof. Dr K. Coninx, Hasselt University, Diepenbeek, BELGIUM, Chair
Prof. Dr A. Cuypers, Hasselt University, Diepenbeek, BELGIUM, Promoter
Prof. Dr J. Vangronsveld, Hasselt University, Diepenbeek, BELGIUM, Co-promoter
Prof. Dr M. Ribas-Carbó, Universitat de les Illes Balears, Palma de Mallorca, SPAIN
Dr I. Florez-Sarasa, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Potsdam-Golm, GERMANY
Prof. Dr N. Verbruggen, Université Libre de Bruxelles, Brussels, Belgium
Prof. Dr F. Van Breusegem, VIB, Ghent University, Ghent, BELGIUM
Dr T. Remans, Hasselt University, Diepenbeek, BELGIUM

WOORD VOORAF

First, I would like to gratefully acknowledge the critical evaluation of my PhD thesis by all members of the jury: Prof. Dr Miquel Ribas-Carbó, Dr Igor Florez-Sarasa, Prof. Dr Nathalie Verbruggen, Prof. Dr Frank Van Breusegem and Dr Tony Remans.

"Keep calm and finish your PhD". De quote die sinds enkele maanden een prominente plaats inneemt boven mijn bureau, op ooghoogte vlak naast mijn laptop. Vandaag is de dag aangebroken, en wat heb ik er naar uitgekeken - ook al was ik niet altijd even kalm ☺. Op 26 augustus 2014 wist ik dat ik exact drie maanden later mijn werk mocht verdedigen. De voorbije vijf jaren zijn zo snel gegaan ("Time flies when you're having fun"), maar het was zo ontzettend hard de moeite waard. Omdat dit voorwoord de enige plek is waar ik lekker lange zinnen kan schrijven, ga ik er wel degelijk gebruik van maken (met alvast mijn oprechte excuses voor enige melodramatiek).

Ik had nooit gedacht dat mijn stages in de master Biomedische Wetenschappen het begin zouden zijn van een geweldig biologisch avontuur. "Teachers plant seeds that grow forever". Ann, wat een geweldige leermeester tijdens het doctoraatsparcours dat ik heb afgelegd! Eens kijken, wat heb ik allemaal geleerd dankzij u? 1: Eerst denken, dan doen. En dan weer denken. 2: Zet het op papier, maak lijstjes en vink dingen (met een dikke vette stift) af ☺. 3: Weeg altijd de voor- en nadelen af voor je een beslissing maakt. 4: Grijp een kans als je ze krijgt. 5: Er zijn echt mensen die hun deur altijd open hebben staan. 6: Vliegangst is overkomelijk (ook zonder gin-tonic). 7: Er is toch iemand op de wereld die oranje Mentos lust. 8: Hoe je studenten warm kan maken voor het "oxidative damage versus signalling" verhaal. 9: Een vat is voor Kempenaars wat een ton is voor Limburgers. 10: Cocktails mengen is geen goed idee, zeker niet op een doordeweekse congresavond. 11. Dat rijmen, zo goed, ik snap nog steeds niet hoe u het doet. 12. Op foto sta ik beter zonder jas, maar ik mag hem dan achteraf zeker niet vergeten! 13: Spiritu Santo is de allerbeste olijfolie ter wereld. Ik denk dat ik nog een tijdje met dit lijstje kan doorgaan, maar wat ik eigenlijk wil zeggen: ik had me geen betere mentor kunnen wensen! Uw onuitputtelijke enthousiasme, gegronde mensenkennis, uw kracht om te blijven relativeren en zeker uw doorzettingsvermogen zullen me altijd bijblijven. Superbedankt Ann, voor alles! Jaco, bedankt voor de ontelbare mailtjes, geruststellende woorden en interessante weetjes (zoals waarom varkensvoer vroeger Cu bevatte ☺). Het is een plezier om samen met u te brainstormen en steeds opnieuw verbaasd te zijn over de wondere wereld van de planten! Tony, dankjewel om je kennis met me te delen! Onze onzekerheden over het normaliseren van genexpressie-resultaten en de "grijze zone" zijn binnenkort voltooid verleden tijd dankzij GrayNorm ☺. Onze kleine brainstormmomenten hebben me meer bijgebracht dan eender welk tekstboek zou kunnen.

Bedankt ook om je inzicht in de "6 G's" met me te delen: zo begrijp ik nu beter hoe een bepaalde geschiedenis de gedachten, gevoelens en het gedrag na een bepaalde gebeurtenis kan beïnvloeden en hoe dit dan gevolgen kan hebben. Sacha, thanks for calming me down during the delayed flight from Croatia to Munich ☺. I appreciate your direct approach, critical eye and indispensable contributions to our group brainstorm sessions.

Heidi en Marijke, vijf jaar geleden begonnen we samen aan ons groot PhD-avontuur. Het was fijn om samen met jullie te groeien van master- tot doctoraatsstudent. Heidi, bedankt voor je luisterend oor en je enthousiasme, over zowel pietluttige kleinigheden als over de belangrijkste zaken in de wereld. Dankjewel dat ik altijd op je kan rekenen, op eender welk moment. Bedankt voor alles wat je me hebt bijgebracht over het mama-zijn, want ook hier heb ik echt veel aan gehad. Marijke, van biomedici naar (bijna) doctoren in de biologie (volgens sommigen is die koerswijziging een waanzinnige vooruitgang ☺). Het was fijn om dit parcours samen af te leggen. Heel veel succes, over een drietal weken is het immers "your time to shine". Je gaat dat schitterend doen, daar ben ik rotsvast van overtuigd. Ook een dikke merci aan Kelly, die ondertussen al twee jaar geleden heeft afgelegd. Je was een goede begeleidster tijdens mijn leerproces in het lab! Ik ben blij dat je een toffe job hebt gevonden! Ook een welgemeende dankjewel aan An! Ik zal je enthousiasme, ijver en goedgemutst karakter nooit vergeten. Heel veel succes met alles wat je in je leven verder onderneemt! Na An ben ik de tweede in een rij van vier (succes Marijke en Kerim!) die de oxidatieve stress groep als doctoraatsstudent zal verlaten. Echter, de opvolging is sowieso verzekerd met Hanne, Heidi en Rafaela en onze nieuwste aanwinsten Sophie, Stefanie, Christophe en Alejandro! Heel veel succes allemaal en keep up the good work! Lieve Hanne, je bent een echte steun en toeverlaat, een prachtpersoon die ik een bijzonder warm hart toedraag. Ongelooflijk hoe je (ondanks je eigen kleine vliegangst) toch op mij wist in te praten in de lucht tussen Brussel en Dublin, zodat we geland waren zonder dat ik er erg in had ☺. Rafaela, although I kind of missed your start in our group at the end of 2012, it was a great pleasure to work with you! Good luck! Sophie, het was fijn je te zien evolueren van master- naar doctoraatsstudent. Bedankt voor je bijdrage in het AOX-verhaal! Ik kijk al uit naar de resultaten rond cadmium en endoreduplicatie, spannend! Stefanie, mijn rechterbuur sinds een paar maanden, wat een plezier om zo'n gedreven dame naast me te zien zitten! Jouw efficiëntie is bewonderenswaardig, dat kan niet anders dan helemaal goed komen! En go go go RootNav, eindelijk geen Optimas meer ☺. Christophe, echt fijn dat je onze groep komt versterken, ook omdat je voor wat mannelijk tegenwicht kan zorgen in onze groep (Kerim heeft geprobeerd, maar kon het helaas niet aan ☺). Alejandro, thanks for making me laugh during these last couple of stressful months, it was such a pleasure to get to know you!

Aan al mijn ex- en huidige bureaugenoten, een oprechte dankjewel om mijn kleine trekjes (lees: soms een klein beetje gek ☺) te verdragen. Kerim, bedankt voor je onmisbare bijdrage in het ethyleen-zijspoor dat we genomen hebben! Ik zal me onze "brainstorm/wash-sessies" nog lang blijven herinneren ☺. Wat ik ook van jou heb geleerd, is dat piekeren eigenlijk volkomen nutteloos is. Een wijze les die veel voor mij betekend heeft. Bedankt en heel veel succes op 26 februari! Bram, bedankt voor al je wijze raad en inzicht, zeker in de menselijke psyche ☺. Van mij moet je uit alle dingen die ik je ooit heb gezegd (en dat zijn er ongetwijfeld heel erg veel) slechts één iets proberen te onthouden: zwart en wit staan je echt niet! Lieve Sarah, wie had ooit gedacht dat we zouden evolueren van twee verre kennissen aan de flow van Brigitte tot collega's en echte maatjes? Waarschijnlijk is dat ook wel een beetje te wijten aan onze vergelijkbare karaktertrekken (hoor ik daar iemand "zotte mussen" zeggen?). Je zal voor altijd een speciaal plekje in mijn hart innemen. Dankzij jou heb ik ook mezelf een heel stuk beter leren kennen. Michiel, nu in het verre Zweden, jij bent de slimste! Ik denk met veel plezier terug aan alle kleine en grote dingen die ik van jou heb geleerd. Je enthousiasme als je op nieuwe dingen uitkwam, is altijd erg aanstekelijk geweest (en nee, ik heb geen sarcasme-bordje nodig ☺). Allebei heel erg bedankt en veel succes met de nieuwe uitdaging!

Ik ben erg blij dat ik deel mag uitmaken van onze fantastische biologiegroep, mede dankzij de geweldige sfeer en groepsdynamiek die er heerst. Vandaar een dikke merci aan alle collega's, al wil ik hier toch enkele personen in het bijzonder vermelden. Karen, dankjewel voor alles wat ik van jou heb mogen leren, zowel op professioneel als persoonlijk vlak. Michelle, ik heb genoten van onze samenwerking tijdens het lesgeven! Veel succes in Londen! Joske, ik bewonder je durf om je hele hebben-en-houden te verhuizen naar Nancy en daar je postdoc-droom na te streven. Bedankt voor je nuchtere kijk op de dingen, die alles zo weer in het juiste perspectief kan plaatsen. En voor ik het vergeet, Bianca, Heidi, Joske en Nele, het is leuk om af en toe even te ontspannen en het doen-en-laten van onze kleine pagadders te bespreken (laat ze ons maar de "mamaatjes" noemen, ik vind dat stiekem niet zo erg ☺). Ann en Carine, wat was het fijn dat mijn eerste bureau vijf jaar geleden zo kort bij jullie stond! Niets is jullie ooit teveel. Ann, als ik aan jou denk, zie ik meteen die ontelbare hoeveelheden kleine *Arabidopsis* zaadjes voor mij (het moeten er zeker en vast miljarden zijn) die jij al gebulkt hebt ☺. En je snelle zaai- en oogsthanden kwamen altijd van pas! Om over de hulp bij de talrijke metaaldoseringen en glutathionmetingen nog maar te zwijgen. Daar is maar één woord voor: SUPER-ANN! Carine, lachend noem ik je wel eens "the godmother" van ons lab. Maar ik meen het ook echt! Jouw kennis van zaken en wil om overal en altijd bij te springen zal ik nooit vergeten. Dankjewel! Brigitte, Ria en Natascha, bedankt om zo nu en dan eens te vragen: "Alles goed?". Dat betekent meer voor mij dan jullie zouden denken.

Natascha, bedankt voor de perfect gesneden en gecontrasteerde EM-coupes! Ook een dikke merci aan Frank om me op weg te helpen aan de microscoop! Bedankt Sascha en Eline, twee medestudenten Biomedische Wetenschappen die het ook in de biologie-richting zijn gaan zoeken ☺. Sascha, wat bewonder ik je kracht en doorzettingsvermogen. Eline, het is fijn zo af en toe iets te horen uit het verre Mol en om samen verwonderd te zijn over de gelijkenissen tussen de effecten van uranium en cadmium in onze geliefkoosde *Arabidopsis* planten. Veel succes verder op het SCK! Dankjewel Nathalie, voor de fijne babbels tijdens mijn occasionele bezoekjes in Mol! Ook een grote dankjewel aan Nele, zeker om ons in te wijden in het endoreduplicatie fenomeen! En voor alle "non-*Arabidopsis*-believers" nog even deze (ik weet het, weer een quote, maar ik ben dan ook een echte addict): "Weeds are flowers too, once you get to know them".

Dan het thuisfront in Neerpelt, om te beginnen mijn ouders. Mama en papa, bedankt om in me te geloven en me alle kansen te geven die me tot op dit punt hebben gebracht. Ook al heb ik mijn perfectionistisch trekje van jou doorgekregen, papa, het heeft me zeker geholpen de voorbije jaren! Bedankt om te luisteren en telkens opnieuw te vragen hoe het ging. Dankjewel voor de ontelbare telefoontjes, sms'jes en jullie onuitputtelijke steun. Ann, samen vorm(d)en we vaak een discussiërend zussenduo, maar weet dat ik er altijd voor je zal zijn. Ik ben fier op wat je hebt bereikt! "Hoe gaat het met de plantjes? Groeien ze nog goed?" Twee van de vragen die steevast bij elk bezoekje aan oma en opa aan bod kwamen ☺. Bedankt voor jullie interesse, steeds opnieuw! Dikke merci ook aan Paulien, jouw lach maakt me steeds vrolijk! Ook mijn schoonouders en schoonfamilie wil ik hier in de bloemetjes zetten en bedanken voor alle kleine en grote dingen. Een woordje van dank ben ik zeker ook verschuldigd aan Karolien en Tineke. Meiden, bedankt om altijd klaar te staan met raad en daad, bedankt om me op tijd en stond even weg te halen van lab/laptopwerk voor de nodige ontspanning, bedankt voor jullie onnoemelijk grote hart en oprechte interesse. Katrien en Katrijn, twee sterke madammen, ik ben blij dat ik jullie heb leren kennen. Bedankt voor de ontelbare toffe avonden, om even stoom te kunnen aflaten en er zo weer volledig voor te kunnen gaan. "True friends bring out the best in you", en dat klopt zeker in jullie geval. Duizendmaal bedankt.

Save the best for last: Roelie. Als er een Nobelprijs zou uitgereikt worden aan de meest geduldige persoon op deze wereld, weet ik absoluut zeker wie deze zou winnen! Sinds het begin van deze uitdaging ben je mijn rots in de soms woelige branding die een doctoraat kan zijn ☺. Maar, "no road is long in good company". Wie zorgde ervoor dat mijn brooddoos 's middags jaloerse blikken opriep? Wie heeft onnoemelijk veel hydrocultuurbuisjes geknipt en plakband rond epjes gedaan? Wie vergezelde me graag om naar mijn plantjes te gaan kijken op zaterdag, weliswaar met een welverdiend blikje cola light/zero achteraf? Wie stelde me altijd gerust?

Wie kon mee denken over de soms verrassende resultaten die ik uitkwam? Wie kalmeerde me als mijn laptop op de meest cruciale momenten besliste om niet meer te reageren? Wie hield me eraan steeds te relativeren? Wie heeft mijn trouwring om half zes 's morgens uit de radiator gehaald en zelfs teruggevonden op de bodem van de Middellandse zee? De lijst van dingen die jij voor mij doet/gedaan hebt, is eindeloos. Tijdens de voorbije vijf jaren hebben we een echt huisje-boompje-beestje parcours afgelegd, met als kers op de taart het wondertje dat we op de wereld getoverd hebben. Roel en Lukas, mijn grote en kleine man, mijn twee persoonlijke superhelden, dankjewel voor jullie onvoorwaardelijke liefde en steun. Deze dag wordt net een beetje specialer, omdat ik hem met jullie mag delen.

Els Keunen - November 2014

SUMMARY

Toxic concentrations of metals such as cadmium (Cd) have been accumulating in soils and water, mainly due to anthropogenic activities such as mining. Environmental Cd exposure is widely recognised as a major health threat to humans, mostly through the consumption of plant-derived food. Plants exposed to Cd show a reduced growth as the metal disrupts physiological processes such as photosynthesis and respiration. At the cellular level, Cd phytotoxicity is intimately linked to an increased generation of reactive oxygen species (ROS), thereby outweighing cellular antioxidants and leading to an oxidative challenge. On the one hand, ROS are able to oxidatively damage cellular constituents. Concurrently, they convey cellular information to activate defence responses finally contributing to plant acclimation during Cd exposure. At the subcellular level, Cd targets plant mitochondria at the level of their electron transport chain (ETC). The resulting increase in mitochondrial ROS levels is potentially involved in retrograde signalling by which mitochondrial signals regulate nuclear gene expression. In addition, mitochondrial respiration supplies cells with ATP and carbon intermediates that need to be adjusted during stress conditions such as Cd exposure. For these reasons, mitochondria are suggested to be involved in Cd stress perception as well as response signalling. Within this framework, transcript levels of the mitochondrial alternative oxidase (AOX) – and more specifically isoform AOX1a – are commonly used as read-out marker for mitochondrial retrograde signalling in plants. Acting besides the cytochrome ETC pathway, AOX directly reduces O_2 to H_2O without contributing to the generation of the proton motive force that ultimately drives ATP synthesis. Expression of AOX genes is triggered by a variety of stressors in different plant species. The functional significance of AOX induction during Cd stress might be directly related to its ability to control mitochondrial ROS production. Indirectly, the cellular energy status may change because of the non-phosphorylating nature of alternative respiration mediated by AOX.

The main objective of the current work was to uncover the involvement of mitochondria and in particular AOX during the Cd-induced oxidative challenge in *Arabidopsis thaliana* using environmentally realistic Cd exposure conditions.

To reveal acclimation-related responses, the applied Cd concentrations should be situated in a sublethal yet toxic range. Two environmentally realistic Cd concentrations (5 or 10 µM) were chronically applied to the roots of *A. thaliana* plants grown in hydroponics. Using an established phenotypic framework methodology, the impact of Cd on the ability of the plants to complete their life cycle was determined. Although vegetative (rosette) growth of Cd-exposed plants was drastically reduced in a dose-dependent manner, both concentrations led to similar reductions in inflorescence height and silique counts. All plants were able to flower and form siliques containing germinative seeds, which confirms the non-lethality of both 5 and 10 µM Cd for hydroponically grown and exposed *A. thaliana* plants **(Chapter 3)**. These concentrations were used throughout the following experiments to study Cd toxicity within a short-term exposure period up to 72 h **(Chapters 4, 5 and 6)**.

To meet the aimed objective, the induction of the mitochondrial alternative respiratory pathway was analysed in *A. thaliana* roots and leaves using a kinetic exposure setup. Responses were studied at transcript and protein levels after 2, 24, 48 and 72 h exposure to 5 or 10 µM Cd. Cadmium-exposed *Arabidopsis* plants were oxidatively challenged at the mitochondrial level, to which they readily responded by inducing *AOX1a* transcription in both roots and leaves. In addition, AOX protein levels were increased within 24 h of Cd exposure. On the other hand, transcript levels of antioxidative enzymes functional in plant mitochondria were generally decreased in roots and only marginally influenced in the leaves. Therefore, the functional significance of AOX induction might be to minimise the extent of Cd-induced ROS production at the mitochondrial level **(Chapter 4)**.

To gain insight into the functional implications of AOX activation during sublethal Cd exposure, responses of wild-type *A. thaliana* plants were compared to those of plants lacking functional AOX1a (*aox1a* knockout mutants) in the following sections **(Chapters 5 and 6)**. First, the involvement of AOX1a in Cd-induced mitochondrial signalling pathways was studied in relation to the oxidative challenge in *A. thaliana* leaves. Within this framework, the involvement of ethylene – either directly or indirectly through NADPH oxidase-dependent ROS

production – in Cd-induced *AOX1* transcriptional activation was demonstrated. Furthermore, AOX is suggested to exert negative feedback on ethylene biosynthesis and/or signalling. In the absence of AOX1a, differences in oxidative stress-responsive transcript and glutathione levels are indicative of an increased oxidative challenge during moderate (*i.e.* 5 µM) and prolonged (72 h) Cd exposure in the leaves. It is therefore suggested that AOX1a acts early in the response to Cd by activating or maintaining a mitochondrial signalling pathway impacting cellular antioxidative defence at post-transcriptional level. In addition, hydrogen peroxide (H_2O_2) might be implicated as retrograde signalling molecule. However, during more severe (*i.e.* 10 µM) Cd exposure, this fine-tuning signalling pathway seemed to be overruled, potentially by an increased generation of ROS and oxidative damage **(Chapter 5)**.

Finally, as mitochondria and AOX are both involved in maintaining metabolic homeostasis during stress conditions, metabolic profiling was performed for roots and leaves of wild-type and *aox1a* knockout plants exposed to 5 or 10 µM Cd during 24 and 72 h. Mitochondrial metabolism, e.g. at the level of the tricarboxylic acid (TCA) cycle, reacted oppositely to sublethal Cd exposure in both tissues. In addition, a reciprocal relationship was established between AOX1a and Cd-induced antioxidative defence mechanisms in *A. thaliana* roots. In leaves, our results confirmed the relationship between ethylene and AOX and also pointed towards the potential involvement of AOX1a in stress-induced respiration based on protein degradation **(Chapter 6)**.

In conclusion, all results support the view that AOX1a modulates the extent of the cellular oxidative challenge in roots and leaves of *A. thaliana* plants exposed to sublethal Cd concentrations within 72 h after the start of exposure. Furthermore, AOX is suggested to be involved in metabolic homeostasis during Cd stress in the leaves. As the maintenance of the mitochondrial ETC and cellular metabolism is suggested to be a central event in stress tolerance, AOX might contribute to plant acclimation to sublethal Cd exposure at the physiological level.

SAMENVATTING

Voornamelijk tengevolge van antropogene activiteiten zoals mijnbouw komen toxische gehalten aan metalen zoals cadmium (Cd) verspreid voor in bodem en water. Mensen worden vooral blootgesteld aan Cd via de consumptie van gecontamineerd plantaardig voedsel. Dit brengt aanzienlijke gezondheidsrisico's met zich mee. Ook voor planten is Cd schadelijk. Planten blootgesteld aan Cd vertonen een verminderde groei door een verstoring van fysiologische processen zoals fotosynthese en respiratie. Op cellulair niveau bestaat er een verband tussen Cd-fytotoxiciteit en een toename in de productie van reactieve zuurstofvormen (ROS), die het overwicht nemen op de antioxidanten met een verstoorde redoxbalans tot gevolg. Enerzijds kunnen ROS oxidatieve schade toebrengen aan cellulaire componenten. Anderzijds kunnen ze ook cellulaire informatie doorgeven en hierdoor verdedigingsmechanismen activeren die bijdragen aan een nieuw evenwicht in planten blootgesteld aan Cd. Op subcellulair niveau vormt de mitochondriale elektronentransportketen (ETK) een belangrijk doelwit tijdens Cd-blootstelling in planten, met een toename in mitochondriale ROS-productie tot gevolg. Deze ROS kunnen fungeren als signaalmoleculen in mitochondriale retrograde signaaltransductie. In dit proces worden signalen vanuit de mitochondriën doorgegeven aan de celkern, waardoor de expressie van nucleaire genen beïnvloed wordt. In de reactie van planten op Cd-stress is een aanpassing van het mitochondriaal energiemetabolisme essentieel. Bovenstaande argumentering leidt tot de hypothese dat mitochondriën betrokken zijn in signaaltransductie, zowel tijdens de perceptie als tijdens de respons van planten op Cd. Een verhoogd expressieniveau van het mitochondriaal alternatief oxidase (AOX) – meer specifiek isovorm AOX1a – wordt algemeen beschouwd als bewijs voor het optreden van mitochondriale retrograde signaaltransductie in planten. Naast de klassieke cytochroom ETK functioneert AOX in een alternatieve ETK door O_2 direct om te zetten tot H_2O, echter zonder bij te dragen aan de protonengradiënt die de synthese van ATP aandrijft. In verscheidene planten is de expressie van genen die coderen voor AOX vaak verhoogd tijdens stress. Het is geweten dat AOX de productie van mitochondriale ROS kan beperken, met mogelijke implicaties tijdens Cd-stress.

Anderzijds kan een activering van AOX de cellulaire energiebalans beïnvloeden omwille van een verminderde ATP-synthese.

De centrale doelstelling binnen deze doctoraatsstudie betreft het ontrafelen van de betrokkenheid van mitochondriën en meer specifiek AOX in de respons van *Arabidopsis thaliana* planten op realistische Cd-blootstelling, voornamelijk in relatie tot de verstoorde redoxbalans. De toegediende Cd-concentraties moeten dus subletaal, maar toch toxisch zijn. Daarom werd de invloed van chronische blootstelling aan twee realistische Cd-concentraties (5 of 10 µM) op het verloop van de levenscyclus van de planten bepaald in een gevalideerd hydrocultuur kweeksysteem via een gevestigde fenotyperingsmethode. De vegetatieve (rozet)groei van Cd-blootgestelde planten verminderde op een dosisafhankelijke wijze. Anderzijds hadden beide Cd-concentraties een gelijkaardig negatief effect op de groei van de bloeisteel en het aantal hauwtjes. Alle planten kwamen tot bloei en produceerden kiemkrachtige zaden. Het subletaal karakter van zowel 5 als 10 µM Cd werd dus bevestigd in dit experiment **(Hoofdstuk 3)**. Daarom werden beide concentraties in de volgende experimenten gebruikt om de effecten van Cd-toxiciteit op korte termijn (binnen een tijdsbestek van 72 uur) te bestuderen **(Hoofdstukken 4, 5 en 6)**.

In een tweede luik werd de inductie van de alternatieve ETK op transcriptioneel en eiwitniveau nagegaan in *A. thaliana* wortels en blaadjes na 2, 24, 48 en 72 uur blootstelling aan 5 of 10 µM Cd. Cadmiumblootstelling veroorzaakte een veranderde redoxbalans op het niveau van de mitochondriën, waarop de planten acuut reageerden door een inductie van *AOX1a* transcriptie in zowel wortels als blaadjes. Ook steeg het AOX-eiwitniveau binnen 24 uur blootstelling aan Cd. Nochtans werd een algemene daling of weinig verandering waargenomen voor wat betreft de expressie van genen die coderen voor mitochondriale antioxidatieve enzymen in respectievelijk wortels en blaadjes. De functionele rol van AOX is dus mogelijk gerelateerd aan het beperken van mitochondriale ROS-productie tijdens Cd-blootstelling **(Hoofdstuk 4)**.

Tot slot werden de functionele implicaties van de activering van AOX nader bestudeerd door de stressresponsen van wild-type *A. thaliana* planten te vergelijken met deze van planten zonder functioneel AOX1a (*aox1a* knockout mutanten) tijdens subletale Cd-blootstelling **(Hoofdstukken 5 en 6)**. Vooreerst werd de rol van AOX1a in signaaltransductie vanuit de mitochondriën en de verstoring van de cellulaire redoxbalans nagegaan in de blaadjes. Hierbij werd aangetoond dat ethyleen – direct of indirect via ROS-productie door NADPH oxidasen – betrokken is bij de transcriptionele activering van *AOX1* genen tijdens Cd-stress. Anderzijds suggereren de data dat AOX een negatieve feedback uitoefent op de biosynthese van ethyleen en/of signaaltransductie gemedieerd door ethyleen. Verder werden in afwezigheid van functioneel AOX1a verschillen geobserveerd wat betreft transcripten van stressmerkers en glutathiongehalten ten opzichte van wild-type planten. Beide observaties wijzen op een versterkte verstoring van de cellulaire redoxbalans in *aox1a* knockout planten blootgesteld aan 5 µM Cd gedurende 72 uur. Er is dus mogelijk een rol weggelegd voor AOX1a in de snelle respons op Cd, meer bepaald via het activeren of onderhouden van een signaal vanuit de mitochondriën. Dit signaal beïnvloedt mogelijk de cellulaire antioxidatieve verdediging op post-transcriptioneel niveau. Waterstofperoxide (H_2O_2) is een kandidaat molecule om het mitochondriaal signaal van AOX door te geven. Tijdens blootstelling aan 10 µM Cd zijn er duidelijk ook andere signalen actief naast AOX, mogelijk als gevolg van een verhoogde productie van ROS en oxidatieve schade aan cellulaire componenten **(Hoofdstuk 5)**.

Aangezien zowel mitochondriën als AOX bijdragen tot de metabole homeostase tijdens stress, werd nagegaan hoe een blootstelling aan 5 of 10 µM Cd gedurende 24 en 72 uur de gehalten aan primaire metabolieten beïnvloedt in wortels en blaadjes van wild-type en *aox1a* knockout planten. Op het niveau van de Krebscyclus reageerden de mitochondriën tegenovergesteld in beide weefsels. Bovendien werd in de wortels een wederzijdse relatie gevonden tussen antioxidatieve verdedigingsmechanismen en AOX tijdens blootstelling aan subletale Cd-concentraties. Tenslotte bevestigen de resultaten in de blaadjes de relatie tussen ethyleen en AOX zoals eerder beschreven. Ook wijzen ze op een

mogelijke rol voor AOX1a in een respiratieketen gekoppeld aan eiwitafbraak **(Hoofdstuk 6)**.

Samengevat kan worden gesteld dat AOX1a een invloed heeft op de mate waarin de cellulaire redoxbalans wordt verstoord door Cd. Dit werd zowel in wortels als blaadjes geobserveerd tijdens subletale Cd-blootstelling binnen een tijdsbestek van 72 uur. Ook is AOX betrokken in het behoud van de metabole homeostase tijdens Cd-stress in de blaadjes. Aangezien het goed functioneren van de mitochondriale ETK alsook een metabole homeostase van cruciaal belang zijn voor Cd-stresstolerantie, blijkt uit onze data dat AOX kan bijdragen aan dit proces op fysiologisch niveau.

INDEX

LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylic acid
ACS	ACC synthase
AOX	Alternative oxidase
APX	Ascorbate peroxidase
AsA	Ascorbate (reduced)
Ca	Calcium
CAT	Catalase
Cd	Cadmium
$CdSO_4$	Cadmium sulfate
CSD	Cu/Zn superoxide dismutase (gene)
Cu	Copper
Cyt c	Cytochrome c
DAB	3,3'-diaminobenzidine
DHA	Dehydroascorbate (oxidised)
DHAR	DHA reductase
EIN2	Ethylene insensitive 2
ETC	Electron transport chain
Fe	Iron
FSD	Fe superoxide dismutase (gene)
GPOD	Guaiacol peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione (reduced)
GSSG	Glutathione disulfide (oxidised)
H_2O_2	Hydrogen peroxide
LOX	Lipoxygenase
MDHA	Monodehydroascorbate
MDHAR	MDHA reductase
Mg	Magnesium
Mn	Manganese
Mn-SOD	Mn superoxide dismutase (enzyme)
MSD	Mn superoxide dismutase (gene)
ND	Alternative NAD(P)H dehydrogenase
Ni	Nickel
NO	Nitric oxide
NTR	NADPH-dependent thioredoxin reductase
$^{\bullet}OH$	Hydroxyl radical
$^1O_2$	Singlet oxygen
$^3O_2$	Molecular oxygen
$O_2^{\bullet-}$	Superoxide radical

PAR	Photosynthetic active radiation
Pb	Lead
PC	Phytochelatin
PCD	Programmed cell death
PCS	Phytochelatin synthase
Prx	Peroxiredoxin
RBOH	Respiratory burst oxidase homologue
ROS	Reactive oxygen species
SHAM	Salicylhydroxamic acid
SOD	Superoxide dismutase
SPOD	Syringaldazine peroxidase
TBA	Thiobarbituric acid
TCA cycle	Tricarboxylic acid cycle
Trx	Thioredoxin
UCP	Uncoupling protein
UPOX	Upregulated by oxidative stress
Zn	Zinc

CHAPTER 1
Introduction

Els Keunen, Tony Remans, Sacha Bohler, Jaco Vangronsveld, Ann Cuypers (2011) Metal-induced oxidative stress and plant mitochondria. International Journal of Molecular Sciences, 12, 6894–6918.

Els Keunen, Darin Peshev, Jaco Vangronsveld, Wim Van den Ende, Ann Cuypers (2013) Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. Plant, Cell and Environment, 36, 1242–1255.

Cadmium (Cd) is a natural element present in the earth's crust as a mineral in combination with elements such as chlorine, oxygen or sulphur. Its natural concentrations however only represent a small fraction of those emanating from anthropogenic activities. On the European continent, the Industrial Revolution started in Belgium in the 18th century. During the following two centuries, the pyrometallurgic industry flourished with active zinc (Zn) smelters in the Campine region. Next to Zn and lead (Pb), the ores contained Cd, which was initially released into the environment during the smelting process. This historical emission is still visible today, with several poorly vegetated areas because of high soil levels of Zn, Pb and Cd. Nowadays, Cd is emitted during mining, the combustion of fossil fuels and incineration of municipal waste. In addition, the agricultural use of metal-containing fertilisers and pesticides has significantly contributed to soil and surface water contamination with Cd. Cadmium compounds are present in e.g. nickel (Ni)-Cd batteries, PVC stabilisers and steel coatings, all potential human exposure sources (Nriagu and Pacyna 1988, Vangronsveld *et al*. 1995, Adriano 2001, ATDSR 2008, WHO 2010).

As opposed to organic contaminants that can be fully metabolised to CO_2 and H_2O, metals such as Cd are not degradable and thus persistent environmental pollutants. Plants form an important bridge between the soil elemental composition and the food chain (Chary *et al*. 2008, Clemens *et al*. 2013), with food as principal source of Cd intake in non-smokers. Additionally, potential human exposure routes are the consumption of contaminated water or the inhalation of polluted air (Figure 1.1). While Cd concentrations in ambient air are usually not high, significant levels are present in tobacco smoke (Clemens *et al*. 2013).

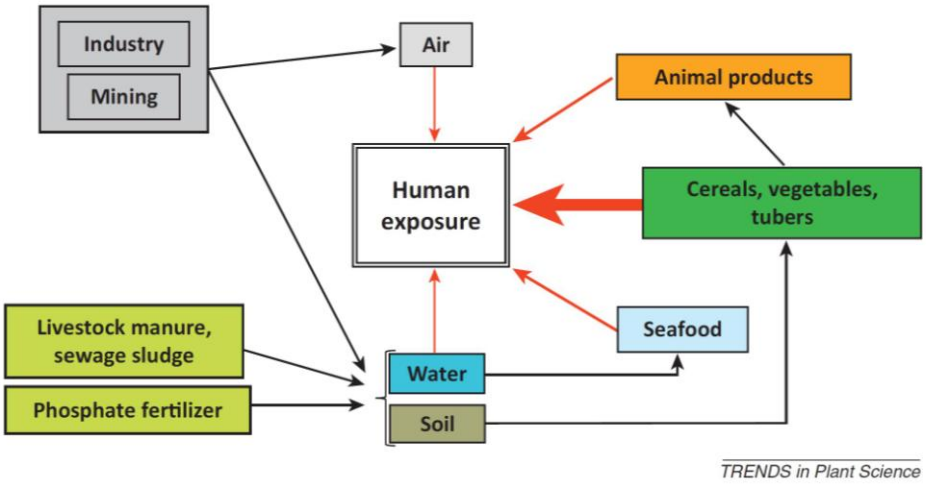


Figure 1.1. *Sources of environmental Cd exposure in the non-smoking general European population (source: Clemens et al. 2013). Industry and mining release Cd into air, water and soil, while the application of fertilisers, livestock manure and sewage sludge contributes to soil and water contamination with Cd. The relative contribution of each human exposure source is indicated by the thickness of the red arrows.*

In the human body, Cd is efficiently retained in the kidney with a biological half-life up to 30 years. Cadmium is nephrotoxic, causes bone damage and is classified as a human carcinogen by the International Agency for Research on Cancer (Järup and Åkesson 2009, Nawrot *et al*. 2010, Nair *et al*. 2013).

1.1. CADMIUM PHYTOTOXICITY

Plant-derived food generally contains higher levels of Cd as compared to meat, eggs, dairy products and fish muscle (Figure 1.1). Namely cereals (wheat and rice), green leafy vegetables (lettuce, spinach and cabbage), potatoes and root vegetables (carrot and celeriac) tend to accumulate Cd (Järup and Åkesson 2009, Nawrot *et al*. 2010). The uptake of Cd by food and feed crops is of great concern as it tremendously increases dietary Cd intake in humans (Figure 1.1) (Clemens *et al*. 2013). A better understanding of metal-induced physiological, cellular and molecular responses in plants will therefore contribute to the development or adjustment of strategies to alleviate Cd-associated risks for human health. For example, Cd-polluted soils could be remediated using plants accumulating Cd in their harvestable parts in a process termed phytoextraction (Vangronsveld *et al*. 2009).

1.1.1. Cadmium uptake and distribution

Plants mainly take up Cd from soil and water, while direct atmospheric uptake is relatively scarce. Two important factors influencing Cd uptake by plants are its concentration and bioavailability, which highly depends on soil pH and organic matter content. As Cd is non-essential, plants do not possess specific uptake mechanisms. Instead, divalent Cd ions enter root cells as "opportunistic hitchhikers" using transport systems of essential divalent nutrients such as calcium (Ca), iron (Fe) and Zn e.g. via zinc-regulated transporter (ZRT)/ iron-regulated transporter (IRT)-like (ZIP) family transporters (Figure 1.2A). This process is strongly stimulated by the highly negative membrane potential of root epidermal cells as well as the presence of intracellular metal binding sites. Once inside the cell, Cd is chelated by phytochelatins (PCs) derived from glutathione (GSH) and subsequently sequestered in the least sensitive cellular sites such as vacuoles via ATP-binding cassette transporters. Furthermore, Cd ions can enter vacuoles via Heavy Metal ATPase 3 (HMA3) and proton/cation exchange transporters, and again be released using Natural Resistance-Associated Macrophage Protein (NRAMP)-type transporters (Figure 1.2A) (Clemens 2006, Van Belleghem *et al*. 2007, Verbruggen *et al*. 2009, Mendoza-Cózatl *et al*. 2011). For long-distance transport, Cd is loaded into the xylem via HMA2/4 transporters (Figure 1.2B) (Verbruggen *et al*. 2009, Wong and Cobbett, 2009). Inside leaf cells, vacuolar sequestration mechanisms are mainly comparable to those operating in roots (Figure 1.2C). Finally, Cd can reach the phloem (Figure 1.2D) and even accumulate in seeds (Figure 1.2E) (Mendoza-Cózatl *et al*. 2011, Khan *et al*. 2014). When Cd concentrations outweigh the capacity of chelation and sequestration mechanisms, free Cd ions can exert their phytotoxic effects (Clemens 2006, DalCorso *et al*. 2008).

To date, nine land plant species are known Cd hyperaccumulators able to accumulate more than 0.01% Cd in their shoot dry weight without toxicity symptoms (Meyer and Verbruggen 2012). Four of them belong to the Brassicaceae family with *Arabidopsis halleri* and *Noccaea caerulescens* as model plants. Studying hyperaccumulators does not only expand our fundamental knowledge on cellular Cd detoxicification mechanisms, but also bears the potential to design or improve phytoremediation strategies (Verbruggen *et al*. 2013).

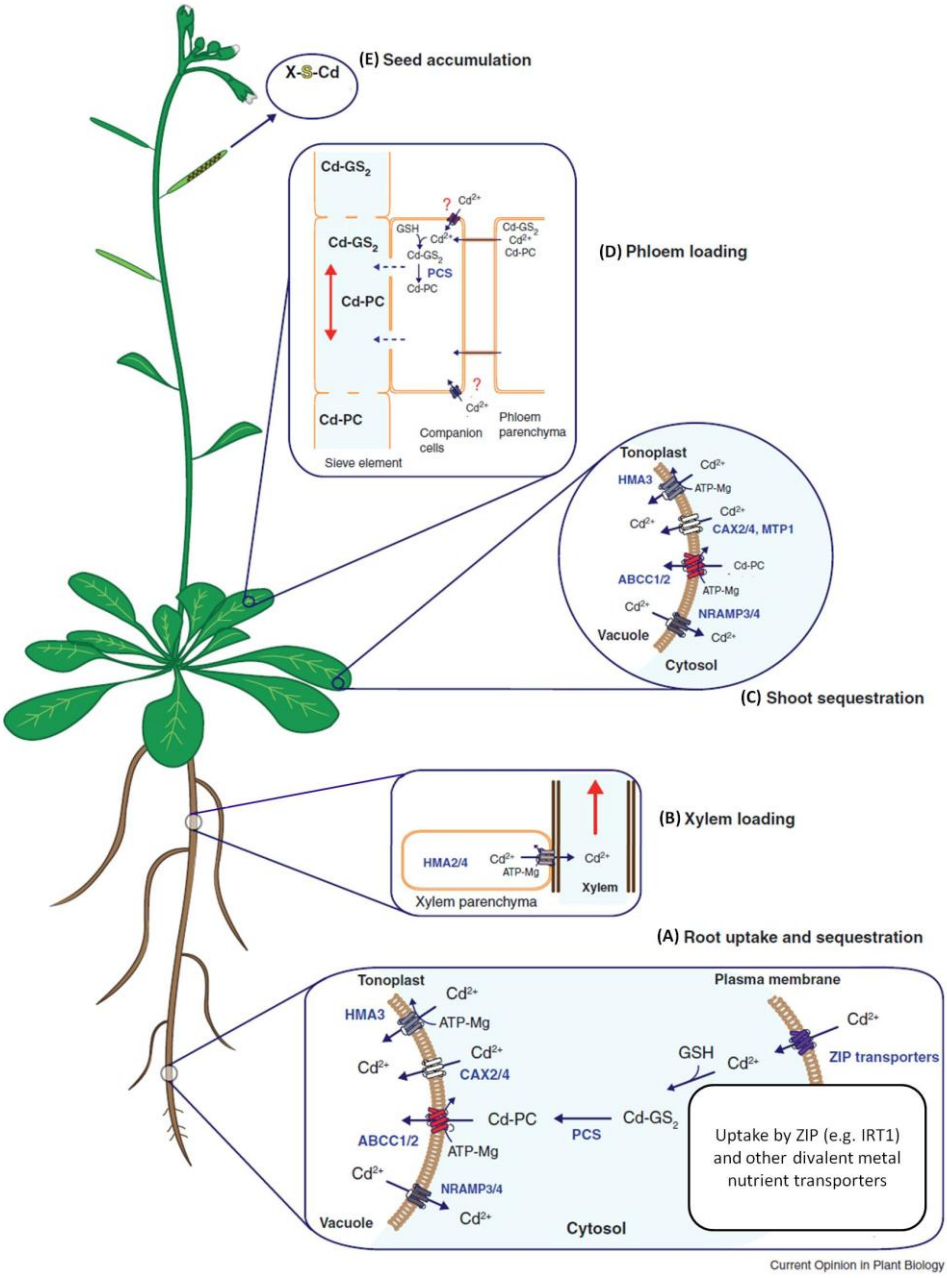


Figure 1.2. *Mechanisms of uptake, sequestration and long-distance transport of Cd in plants (adapted from: Mendoza-Cózatl et al. 2011). (A) Cadmium (Cd^{2+}) uptake at the root level occurs via members of the ZRT, IRT-like protein (ZIP) family or other divalent metal nutrient transporters. In the cytosol, phytochelatin (PC) synthesis starting from glutathione (GSH) is catalysed by phytochelatin synthase (PCS), leading to vacuolar sequestration of Cd-PC complexes by ATP-binding cassette (ABC) transporters (ABCC1/ABCC2).*

Figure 1.2 (continued). *(A) Using Heavy Metal ATPase 3 (HMA3) and proton/cation exchange transporters (CAX-type), Cd^{2+} can also be transported into vacuoles. Finally, Natural Resistance-Associated Macrophage Protein (NRAMP)-type transporters mediate vacuolar release of Cd^{2+} into the cytosol. (B) Via HMA2 and HMA4, Cd^{2+} is loaded into the xylem and transported from root to shoot. (C) In leaves, vacuolar sequestration mechanisms are mainly comparable to those functional in roots. (D) If Cd is not completely sequestered in leaf cell vacuoles, it can reach the phloem parenchyma and companion cells via plasmodesmata (symplastic transport). Because of the high permeability of companion cells and sieve element plasmodesmata, Cd bound to GSH or PCs may enter the phloem stream symplastically. (E) In seeds, Cd is mainly bound to thiol-containing compounds.*

1.1.2. Mechanisms of Cd phytotoxicity

Except for its incorporation in carbonic anhydrase of many marine diatoms under Zn limiting conditions (Lane and Morel 2000), Cd has no known function in plant life and development. Instead, it negatively affects photosynthesis, respiration and the uptake of nutrients and H_2O, thereby significantly reducing plant growth and productivity (DalCorso *et al*. 2008).

An important underlying cause of Cd phytotoxicity is its chemical similarity to essential nutrients such as Ca, Fe and Zn. This feature enables Cd to displace essential cations from their specific binding sites in functional and structural proteins. For example, Cd replaces Ca in the reaction centre of photosystem II (PSII) and thereby inhibits PSII photoactivation (Faller *et al*. 2005). In addition, its high affinity for sulfhydryl groups causes Cd to target protein thiol groups, which might interfere with cellular metabolism. The same characteristic underlies Cd chelation mechanisms as the metal is strongly bound to the cysteine group of GSH, which is the precursor for PC synthesis (Cuypers *et al*. 2009). Finally, although Cd is not redox-active, its phytotoxicity is closely linked to an enhanced generation of reactive oxygen species (ROS) and the resulting oxidative challenge at organellar and cellular levels (Sharma and Dietz 2009, Cuypers *et al*. 2012).

1.2. CADMIUM-INDUCED OXIDATIVE CHALLENGE IN PLANTS

Oxidative challenge refers to a cellular state of imbalance between pro- and antioxidants in favour of the former. Reactive oxygen species (ROS) represent major pro-oxidants in plant cells. Starting from atmospheric oxygen ($^3O_2$), an input of energy generates singlet oxygen ($^1O_2$). Subsequent univalent reduction of O_2 generates superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) or

hydroxyl radicals (•OH) respectively (Gechev *et al*. 2006, Halliwell 2006). Under optimal physiological conditions, ROS are constantly produced as by-products of aerobic metabolism in chloroplasts, mitochondria and peroxisomes. However, their production is tightly controlled and kept at a low level by the antioxidative defence network of plant cells. This system consists of enzymes neutralising $O_2^{\bullet-}$ and H_2O_2 such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD), complemented by metabolites such as ascorbate (AsA) and GSH (Figure 1.3). All subcellular compartments contain specific antioxidative enzymes and metabolites maintaining the cellular redox balance within certain limits (Mittler *et al*. 2004, Gechev *et al*. 2006, Miller *et al*. 2010). However, under abiotic stress conditions such as Cd exposure, the equilibrium between ROS production and detoxification is disturbed thereby leading to an oxidative challenge.

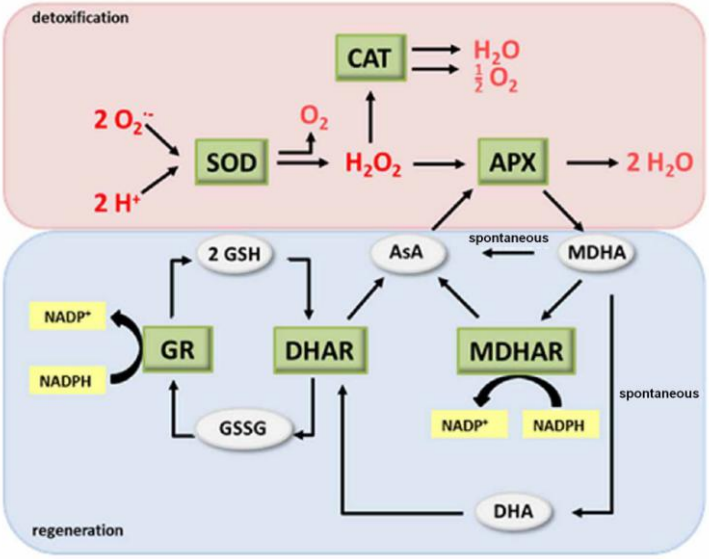


Figure 1.3. *Simplified overview of the components involved in plant antioxidative defence (adapted from: Groß et al. 2013). Superoxide dismutase (SOD) detoxifies $O_2^{\bullet-}$, resulting in H_2O_2 formation which is subsequently neutralised by catalase (CAT), ascorbate peroxidase (APX) or other enzymes. Via APX, ascorbate (AsA) is oxidised to monodehydroascorbate (MDHA) and dehydroascorbate (DHA). While MDHA is regenerated via MDHA reductase (MDHAR), DHA is reduced to AsA via DHA reductase (DHAR) using GSH as electron donor. In the latter reaction, GSH is oxidised to glutathione disulfide (GSSG) and back-reduced by glutathione reductase (GR). Both MDHAR and GR use NADPH as reducing equivalent.*

1.2.1. Cadmium-induced ROS production via indirect mechanisms

As non-redox-active metal, Cd is unable to generate ROS directly. However, it is able to replace Fe in various proteins, thereby increasing cellular levels of free redox-active metals. In turn, these metals directly contribute to ROS production via Fenton and Haber-Weiss reactions (Figure 1.4) (Schützendübel and Polle 2002, Cuypers *et al*. 2010).

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

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

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

Figure 1.4. *Fenton and Haber-Weiss reactions. Oxidised transition metal ($M^{(n)}$), reduced transition metal ($M^{(n-1)}$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and hydroxide ion (OH^-).*

In addition, Cd exposure depletes the cellular GSH pool because of PC formation essential in metal chelation and sequestration (Figure 1.2). However, GSH is also involved in detoxification of pro-oxidants and its exhaustion might thus increase ROS production (Schützendübel and Polle 2002, Jozefczak *et al*. 2014). Cadmium exposure has also been related to the inhibition of enzymes functioning in the cellular antioxidative defence network (Schützendübel and Polle 2002). Besides these effects, Cd can also stimulate enzymatic and organellar ROS production as discussed in the following sections.

1.2.2. Cadmium-mediated enzymatic generation of excess ROS

At the plasma membrane, NADPH oxidases or respiratory burst oxidase homologues (RBOHs) are considered to be the engines of ROS production in response to biotic and abiotic stress such as heat, drought or salinity in plants (Suzuki *et al*. 2011). They catalyse the reduction of O_2 to extracellular $O_2^{\bullet -}$ using intracellular NADPH-derived electrons. It has been shown that Cd might directly stimulate NADPH oxidase activity by mimicking Ca. Calcium homeostasis is closely related to NADPH oxidase-dependent ROS production via a positive feedback amplification mechanism. Indeed, Ca binds EF-hand motifs in the cytosolar N-terminal domain of NADPH oxidases, finally resulting in their activation and ROS production. Subsequently, this ROS may activate Ca channels (Wong *et al*. 2007, Suzuki *et al*. 2011). It has also been shown that

gene expression of RBOH family members increased in roots and leaves of Cd-exposed *Arabidopsis thaliana* plants (Remans *et al*. 2010). Besides NADPH oxidases, lipoxygenases (LOXs) represent another enzymatic source of ROS in plants. Via the dioxygenation of polyunsaturated fatty acids (PUFAs), LOXs produce hydroperoxy fatty acids. Transcript levels of several LOX isoforms were enhanced in Cd-exposed *A. thaliana* roots and leaves (Remans *et al*. 2010) and a clear role has been appointed to LOX1 in Cd-induced stress responses (Keunen *et al*. 2013). Excessive LOX activity might lead to enhanced lipid peroxidation as demonstrated in Cd-exposed barley root tips (Tamas *et al*. 2009).

1.2.3. Cadmium-mediated organellar ROS generation

In addition to its influence on enzymatic pathways, Cd increases ROS production in subcellular organelles such as chloroplasts, mitochondria and peroxisomes, which together constitute the predominant sources of ROS production in plants. Their highly oxidising nature and the presence of electron transport chains (ETCs) in chloroplasts and mitochondria makes both organelles a preferential site for stress-induced ROS production (Halliwell 2006, Sharma and Dietz 2009). In chloroplast thylakoids, the reaction centres of photosystem I (PSI) and PSII account for a large share of total ROS levels produced in plant cells (Asada 2006), which can be increased by Cd exposure (Cuypers *et al*. 2012 and references therein). In non-photosynthetic cells or in the dark, mitochondria constitute the main origin of ROS because of electron leakage at the level of complexes I and III in the respiratory ETC. This too can be enhanced by Cd as will be discussed in section 1.3.2. Similar to chloroplasts and mitochondria, peroxisomes produce ROS as by-products of their physiological oxidative metabolism. Although a relationship has been established between ROS and peroxisomal dynamics during Cd exposure, its biological significance remains to be revealed (Rodríguez-Serrano *et al*. 2009).

1.2.4. Reactive oxygen species – friend or foe?

In plant biology, it is now widely accepted that ROS constitute an ambiguous role during stress responses (Dat *et al*. 2000). Being toxic molecules, they are able to oxidatively injure cells (Møller *et al*. 2007). However, they are also key signalling regulators of defence pathways leading to cellular protection and/or

acclimation (Mittler *et al*. 2004, Gechev *et al*. 2006, Petrov and Van Breusegem 2012). The balance between both outcomes is delicate and requires tight control of accumulating ROS levels during abiotic stress in plants (Miller *et al*. 2008, 2010). As $O_2^{\bullet-}$ and H_2O_2 are less reactive than $^{\bullet}OH$ reacting with every molecule in its neighbourhood within a short half-life of 1 ns (Møller *et al*. 2007), plant cells would benefit from higher concentrations of $^{\bullet}OH$ scavengers exactly at positions where $^{\bullet}OH$ radicals are potentially generated and could cause substantial damage (e.g. in the vicinity of membranes). Indeed, $^{\bullet}OH$ radicals can initiate membrane lipid peroxidation by abstracting a hydrogen atom from the side chain of PUFAs. This further generates multiple lipid peroxides in a chain reaction, affecting membrane fluidity and functioning (Gill and Tuteja 2010). Both $^{\bullet}OH$ and $^1O_2$ are able to attack plant DNA, which finally affects growth and development in various ways (Møller *et al*. 2007). In addition, ROS and/or its by-products are able to covalently modify and oxidise proteins, for which protein carbonylation is a widely accepted marker (Ghezzi and Bonetto 2003). Common targets are sulphur-containing amino acids such as methionine or thiol groups (Gill and Tuteja 2010, Jacques *et al*. 2013). Protein oxidation can serve as alarm signal to initiate or propagate plant responses to abiotic stress (Møller and Kristensen 2004). Therefore, oxidative damage is but one side of the coin.

Conversely, it is generally acknowledged that ROS themselves can act as signalling components mediating plant abiotic stress responses (Dat *et al*. 2000, Gechev *et al*. 2006). Next to their versatile properties, mobility and the delicate balance between production and scavenging, they are intimately related to several signalling and redox networks (Mittler *et al*. 2011). In addition, it becomes increasingly clear that ROS and/or oxidative stress-induced secondary signals are involved in transmitting organelle-specific information to the nucleus during abiotic stress. Galvez-Valdivieso and Mullineaux (2010) recently reviewed the role of ROS in chloroplastic retrograde signalling. Mitochondrial ROS are also suggested to participate in signalling starting from this organelle, for example during metal stress (Yamamoto *et al*. 2002, Rhoads and Subbaiah 2007). Recently, Suzuki *et al*. (2012) reviewed the intense relationship between both organelles in stress-induced redox signalling throughout the plant cell, emphasising the importance of studying plant abiotic stress responses simultaneously in different organelles.

1.3. MITOCHONDRIA AND THE CADMIUM-INDUCED OXIDATIVE CHALLENGE

Chloroplasts are intensively studied in the light of photosynthesis and its accompanying ROS production under metal stress conditions (Kučera *et al*. 2008). However, research has extended to plant mitochondria in recent years (Noctor *et al*. 2007, Sharma and Dietz 2009). Although mitochondria were long considered secondary to chloroplasts as cellular powerhouses with lower ROS levels as compared to other organelles, this view has changed as crosstalk and acclimation between mitochondria and other organelles appears increasingly vital for an integrated cellular energy and redox metabolism and (stress) signalling (Noctor *et al*. 2007, Suzuki *et al*. 2012, Schwarzländer and Finkemeier 2013, Ng *et al*. 2014).

1.3.1. The unique demands placed on plant mitochondria

Mitochondria are the principal organelles performing plant aerobic respiration. In this process, organic acids are oxidised to CO_2 and H_2O in the tricarboxylic acid (TCA) cycle in the mitochondrial matrix, thereby fuelling electrons from reducing NADH (and $FADH_2$) equivalents to O_2 via the respiratory ETC present in the inner mitochondrial membrane (Figure 1.5A). This electron transfer process is coupled to the synthesis of energy in the form of ATP in a process termed oxidative phosphorylation (Sweetlove *et al*. 2007, Millar *et al*. 2011).

In addition to respiration, plant mitochondria play a role in the metabolism of diverse amino acids, vitamins and lipids essential for organellar biogenesis and maintenance. The relative importance of these processes depends on the cell type and developmental stage. Studying the responses of plant mitochondria to metal stress implies the need to fractionate the responses in different plant organs. In leaves, the cellular environment of plant mitochondria is rather distinctive as compared to animal cells due to the presence of photosynthesis-derived O_2 and carbohydrates. In plant roots however, a different cellular environment as compared to the leaves will definitely influence the response of mitochondria to excess metals (Noctor *et al*. 2007, Sweetlove *et al*. 2007). It is noteworthy to mention that plant mitochondria differ from their animal counterparts in that the former function in distinct processes such as photorespiration and that they possess unique components (alternative oxidase (AOX), alternative NAD(P)H dehydrogenases (NDs) and uncoupling

proteins (UCPs), Figure 1.5B) (Dutilleul *et al*. 2003, Noctor *et al*. 2007, Sweetlove *et al*. 2007). Electrons can pass through either the 'standard' cytochrome pathway to complex IV (cytochrome c oxidase) (Figure 1.5A) or through the alternative pathway to the cyanide-insensitive AOX (Figure 1.5B). Although this alternative route does not contribute to ATP synthesis, AOX is currently considered both a target and regulator of stress responses in plants (sections 1.3.3.1. and 1.4.3.) (Rhoads *et al*. 2006, Van Aken *et al*. 2009, Vanlerberghe 2013).

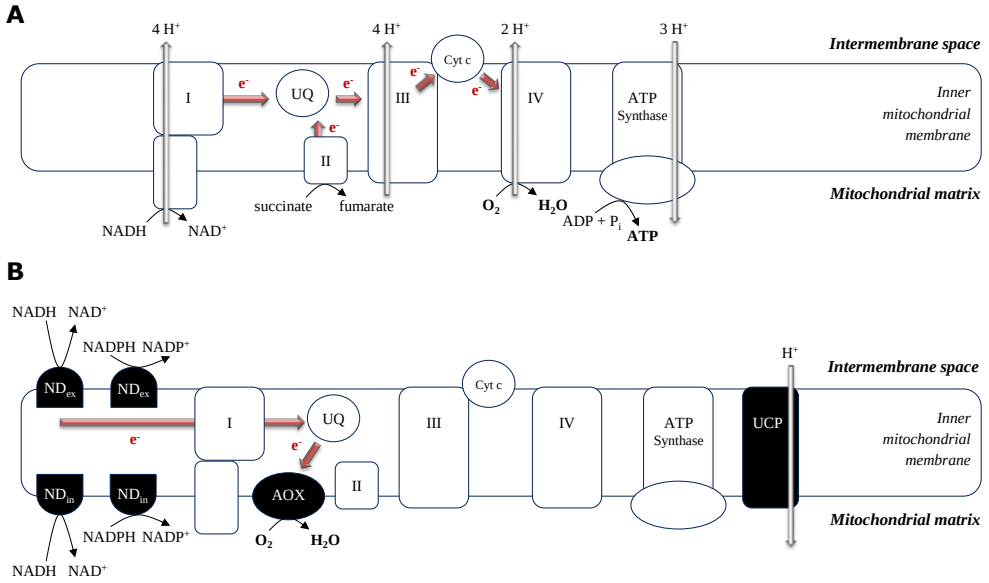


Figure 1.5. *Simplified overview of the components involved in conventional and alternative reactions of mitochondrial electron transport and oxidative phosphorylation in plants. (A) In the 'standard' cytochrome pathway, electrons pass from respiratory complexes I (NADH dehydrogenase) and II (succinate dehydrogenase) to the electron carrier ubiquinone (UQ). Via complex III (ubiquinol-cytochrome bc_1 reductase) and cytochrome c (Cyt c), O_2 is ultimately reduced to H_2O at the level of complex IV (cytochrome c oxidase). The ATP synthase complex catalyses the formation of ATP in the mitochondrial matrix driven by the proton gradient resulting from electron transfer. (B) In addition, plant mitochondria contain an alternative pathway consisting of a non-proton-pumping alternative oxidase (AOX) as well as alternative NAD(P)H dehydrogenases (NDs) on either the external (ND_{ex}) or internal (ND_{in}) side of the inner mitochondrial membrane. Electrons are passed from the alternative NDs to UQ and directly to AOX reducing O_2 to H_2O. Uncoupling proteins (UCPs) are able to dissipate the proton electrochemical gradient over the inner membrane created by the transfer of electrons, thereby acting as an alternative path to mitochondrial oxidative phosphorylation.*

1.3.2. Exposure to Cd increases ROS generation in plant mitochondria

Under normal conditions, the mitochondrial ETC and ATP synthesis are tightly coupled. However, plants suffering from abiotic stress often show an over-reduction of electron carriers such as ubiquinone, causing electron leakage from the system. These electrons possess a sufficient amount of free energy to directly reduce molecular O_2, with increased production of ROS such as $O_2^{\bullet-}$ and H_2O_2 as unavoidable by-products of aerobic metabolism (Møller 2001, Rhoads *et al*. 2006, Amirsadeghi *et al*. 2007, Sweetlove *et al*. 2007, Blokhina and Fagerstedt 2010, De Gara *et al*. 2010). The known sites of ROS production in the mitochondrial ETC are complexes I and III (for reviews, see Møller 2001 and Rhoads *et al*. 2006), where $O_2^{\bullet-}$ is formed and subsequently dismutated to H_2O_2. The uncharged H_2O_2 molecule is able to penetrate membranes and has a longer half-life as compared to $O_2^{\bullet-}$. Both properties make H_2O_2 an ideal candidate for ROS signalling (Petrov and Van Breusegem 2012), for example from mitochondria to other organelles (Sweetlove *et al*. 2007). However, H_2O_2 can also react with reduced Fe and Cu in the mitochondrion itself to produce highly toxic $^{\bullet}OH$ radicals and cause oxidative damage to mitochondrial proteins, lipids and DNA (Rhoads *et al*. 2006, Blokhina and Fagerstedt 2010, Tan *et al*. 2010).

Similar to animals (Cuypers *et al*. 2010), the plant mitochondrial ETC is also considered to be an important target of Cd toxicity (Verbruggen *et al*. 2009). Heyno *et al*. (2008) demonstrated a fast Cd-induced stimulation of ROS generation inside root cells, mainly originating from the mitochondrial ETC. Exposure to Cd impairs proper mitochondrial functioning partly by affecting the organellar redox balance as shown by Smiri *et al*. (2010) in germinating pea seeds. Bi *et al*. (2009) confirmed ROS production in mitochondria prior to chloroplasts in combination with altered mitochondrial distribution and mobility patterns in Cd-exposed *A. thaliana* protoplasts (Table 1.1).

Table 1.1. *Exposure to Cd has consequences for plant mitochondria at different levels. The effects of Cd are shown and categorised based upon the experimental setup used (isolated mitochondria (A), cell cultures/protoplasts (B) or intact plants (C)). Exposure to Cd imposes a mitochondrial oxidative challenge characterised by an increased ROS production and altered antioxidative defence. This oxidative challenge is most likely the result of Cd-induced ETC dysfunction at the level of the cytochrome pathway. In addition, Cd is able to activate the alternative respiratory pathway at different levels, but also induce mitochondrial damage versus signalling and defence (e.g. programmed cell death) responses. Cadmium-induced responses related to plant mitochondria are described schematically in the column 'Observations'. Abbreviations: GR, glutathione reductase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; MDHAR, monodehydroascorbate reductase; $O_2^{\bullet-}$, superoxide; PCD, programmed cell death; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.*

A. CADMIUM-INDUCED RESPONSES IN ISOLATED MITOCHONDRIA					
Concentration	**Exposure time**	**Setup**	**Species**	**Observations**	**Reference**
10-30 µM	30 min	Exposure after isolation out of tubers	*Solanum tuberosum*	↑ ROS production ($O_2^{\bullet-}$ and H_2O_2)	Heyno *et al*. 2008
5 mM	12 to 120 h	Isolation after exposing germinating seeds	*Pisum sativum*	↓ glutaredoxin, GR, GSH	Smiri *et al*. 2010
B. CADMIUM-INDUCED RESPONSES IN CELL CULTURES OR PROTOPLASTS					
Concentration	**Exposure time**	**Setup**	**Species**	**Observations**	**Reference**
20 µM	5 h	Protoplasts	*Arabidopsis thaliana*	↑ H_2O_2 in mitochondria prior to chloroplasts mitochondrial clustering and restricted movement	Bi *et al*. 2009
0.5-2-5-20-50-200 µM	24 h	Cell culture	*Arabidopsis thaliana*	↑ MDHAR, peroxiredoxin	Sarry *et al*. 2006
100 or 150 µM	3 days	Cell culture	*Arabidopsis thaliana*	↑ PCD	De Michele *et al*. 2009
3 mM	1 h	Cell culture	*Nicotiana tabacum*	↑ $O_2^{\bullet-}$	Garnier *et al*. 2006
C. CADMIUM-INDUCED RESPONSES *IN PLANTA*					
Concentration	**Exposure time**	**Setup**	**Species**	**Observations**	**Reference**
30-60-100 µM	Up to 10 days	Roots and leaves	*Hordeum distichum*	↓ respiration (O_2 uptake) ↑ alternative respiratory pathway (SHAM)	Garmash and Golovko 2009

In addition to ROS, plant mitochondria are also able to produce nitric oxide (NO), a free radical that affects the activity of mitochondrial ETC and matrix enzymes and transcriptionally upregulates AOX (Igamberdiev *et al*. 2014). A potential role for NO during Cd stress responses in plants is suggested by De Michele *et al*. (2009), demonstrating the involvement of NO in Cd-induced programmed cell death (PCD) of *A. thaliana* suspension cultures. Arasimowicz-Jelonek *et al*. (2011) discussed the mode of action of NO during Cd stress in plants, with a potential intense relationship between NO and ROS signalling.

1.3.3. Mechanisms to control mitochondrial ROS production during Cd exposure

Plants contain a dynamic antioxidative defence network to counterbalance the accumulation of ROS, thereby limiting their detrimental effects while still allowing redox signalling throughout the plant (Rhoads *et al*. 2006, Blokhina and Fagerstedt 2010). During environmental stress conditions such as Cd exposure, an integrated antioxidative response in and between different cellular organelles and compartments is required to locally act against ROS production (Figure 1.3) (Millar *et al*. 2001, Blokhina and Fagerstedt 2010). Sweetlove *et al*. (2002) studied the impact of oxidative stress induced by H_2O_2, menadione (an intracellular $O_2^{\bullet-}$ generator) or antimycin A (an inhibitor of respiratory complex III) in *Arabidopsis* cells and provided direct evidence for plant mitochondria using an array of enzymes to detoxify ROS (thioredoxin-based redox pathway) or repair oxidative stress-induced damage (protein disulphide isomerase). Based on the fact that Cd induces a mitochondrial oxidative challenge, it could be rationalised that similar mechanisms may be involved to counterbalance the accumulation of ROS. In the following paragraphs, the potential mechanisms exploited by plant mitochondria to avoid or detoxify ROS are discussed.

1.3.3.1. Avoidance of mitochondrial ROS production at the ETC level as a first line of defence

Stress-induced over-reduction of ETC components and resulting electron leakage from the system is a principal cause of mitochondrial ROS production. Plant mitochondria contain energy-dissipating systems able to regulate the mitochondrial membrane potential, thereby decreasing mitochondrial ROS

production due to ETC over-reduction. However, one must keep in mind that these systems are not able to prevent damage by cytosolic ROS diffusing into the mitochondria (Navrot *et al*. 2007). Van Dongen *et al*. (2011) recently reviewed the important role of alternative pathways regulating plant respiration. These pathways ensure metabolic adaptation to hypoxia or altered O_2 availability, which could also indicate their importance during Cd stress conditions in plants.

Plant mitochondria contain several proteins present in the vicinity of the "classical" ETC components, which can alleviate the degree of coupling between electron transport and ATP synthesis. They bypass ETC complexes by diverting electrons from the primary cytochrome c pathway, while energy is dissipated as heat. The first enzyme to be discussed in the context of this alternative pathway is AOX, a terminal oxidase accepting electrons directly from ubiquinone and thereby bypassing complexes III and IV (Figure 1.5B). Since it was first suggested by Purvis and Shewfelt (1993), several studies confirmed that AOX prevents mitochondrial oxidative stress. Maxwell *et al*. (1999) demonstrated a direct link between functional AOX levels and mitochondrial ROS production in *Arabidopsis* cells, thereby confirming the ability of AOX to reduce mitochondrial ROS levels. Recently, Cvetkovska and Vanlerberghe (2012) established for the first time that a lack of AOX increases steady-state *in planta* mitochondrial $O_2^{\bullet-}$ and cellular NO concentrations in tobacco leaves. Exposure to Cd has been shown to affect the alternative respiratory pathway mediated by AOX at different levels. In the protist *Euglena gracilis*, Cd stress led to an increased AOX content and capacity (Castro-Guerrero *et al*. 2008). This was also demonstrated in barley plants, where Cd exposure altered the contribution of the alternative respiratory pathway to total respiration as measured by O_2 uptake in the presence of the AOX inhibitor salicylhydroxamic acid (SHAM). The authors have shown strong effects of high Cd concentrations on total and alternative respiratory rate and suggested the activated alternative respiration to act as a homeostasis mechanism in Cd-stressed root cells (Garmash and Golovko 2009). In a more environmentally realistic setup, Finkemeier *et al*. (2005) have shown that alternative respiration of *A. thaliana* roots exposed to 10 µM $CdCl_2$ for one week increased up to 40% as compared to 20% under control conditions.

From these observations, it is clear that AOX activation could modulate the extent of Cd-induced ROS production in plant mitochondria.

In addition to AOX, plant mitochondria contain alternative NDs able to oxidise cytosolic or matrix NADH/NADPH, thereby bypassing complex I and reducing ubiquinone without pumping protons across the inner membrane (Figure 1.5B) (Rasmusson *et al*. 2008). Although co-regulated expression patterns for several members of the AOX and alternative ND families were detected under multiple stress conditions affecting mitochondrial respiration (Clifton *et al*. 2005, Rasmusson and Møller 2011), more research is needed to explore the potential role of alternative NDs during metal stress in plants.

Mitochondrial UCPs catalyse a proton leak that dissipates the proton electrochemical gradient over the inner mitochondrial membrane, thereby shortcutting the ATP synthase complex and thus oxidative phosphorylation (Figure 1.5B) (Vercesi *et al*. 2006). A number of observations suggest a role for UCPs mediating cellular tolerance to oxidative stress (Nogueira *et al*. 2011). Superoxide (Considine *et al*. 2003) and lipid peroxidation products (Smith *et al*. 2004) stimulate UCP activity in plant mitochondria. The expression of genes coding for UCP is induced by low temperature conditions (Laloi *et al*. 1997) and treatment with H_2O_2 (Desikan *et al*. 2001, Brandalise *et al*. 2003). Overexpressing a gene coding for UCP conferred tolerance to oxidative stress in rice (Ozawa *et al*. 2006) and lack of UCP induced localised oxidative stress in *Arabidopsis* (Sweetlove *et al*. 2006). In durum wheat mitochondria, it was shown that drought stress induced ROS production, which further promoted UCP activity. Thereby, the authors validated a feedback link between stress-induced mitochondrial ROS production and UCP-mediated inhibition of further ROS accumulation (Pastore *et al*. 2007, Blokhina and Fagerstedt 2010). As Cd is able to induce mitochondrial ROS production (section 1.3.2.), a role for UCP is plausible and further research should be conducted in this area.

Although both AOX and UCP activity confer dissipation of energy as heat, they respond to different environmental stimuli. Rasmusson *et al*. (2009) suggested AOX activity to be involved in the acute response to ETC over-reduction, while UCP could become important during prolonged mitochondrial oxidative stress based on their direct versus indirect effect on the transfer of electrons. In addition, the AOX enzyme can be inhibited by lipid peroxidation products, while

UCP is stimulated by $O_2^{\bullet-}$ (Fernie *et al*. 2004, Rasmusson *et al*. 2011), thereby signifying the role both enzymes can play in the Cd-induced oxidative challenge as mentioned above.

1.3.3.2. Mitochondrial enzymes and metabolites involved in the detoxification of mitochondrial ROS

Once formed, $O_2^{\bullet-}$ radicals are rapidly dismutated to H_2O_2 via the manganese superoxide dismutase (Mn-SOD) enzyme present in the mitochondrial matrix (Alscher *et al*. 2002, Navrot *et al*. 2007). It was shown that *MSD1* (Mn-SOD) transcript levels were only slightly affected by Cd exposure in *A. thaliana* plants (Cuypers *et al*. 2011). Using transgenic rice plants overexpressing SOD genes at different subcellular locations, Li *et al*. (2013) have shown that only the mitochondrial SOD attenuated the level of *AOX1* gene induction during cold, drought and salinity stress. They suggest that mitochondrial $O_2^{\bullet-}$, of which the levels are directly controlled by Mn-SOD, is involved in *AOX* induction during stress.

In order to provide optimal defence against $O_2^{\bullet-}$-derived H_2O_2 production, Mn-SOD must act in concert with H_2O_2-scavenging components. An important system removing H_2O_2 is the AsA-GSH cycle comprised of four enzymes (APX, DHAR, MDHAR and GR) together with AsA and GSH that are regenerated using NADPH equivalents (Mittler *et al*. 2004). Jiménez *et al*. (1997) demonstrated the presence of this cycle in plant mitochondria and several cycle enzymes were shown to be dually targeted to mitochondria and chloroplasts (Chew *et al*. 2003). In general, several reports demonstrated that Cd affects the plant AsA-GSH cycle at both enzyme and metabolite levels (Table 1.1, Seth *et al*. (2012) and references therein) and mitochondria show an interesting link to this cycle as they harbour the last enzyme in the AsA biosynthesis pathway. In this final step, L-galactono-γ-lactone (GL) is converted to AsA by the membrane-bound GL dehydrogenase (GLDH), which uses cytochrome c as an electron acceptor and thereby donates electrons to the ETC (Bartoli *et al*. 2000, Blokhina and Fagerstedt 2010). Zhao *et al*. (2005) have demonstrated a protective role for GL during Cd stress in winter wheat. Application of this AsA precursor lowered Cd-induced H_2O_2 production and increased POD activities (Zhao *et al*. 2005).

In addition to AsA biosynthesis, it was shown that AsA regeneration from dehydroascorbate (DHA) is also coupled to the plant mitochondrial ETC, presumably at the level of complex II *i.e.* succinate dehydrogenase (Szarka *et al*. 2007, Blokhina and Fagerstedt 2010). As Cd strongly affects this complex (Reese and Roberts 1985, Garmash and Golovko 2009), it can also influence the rate of AsA reduction when the latter functions as an important antioxidant.

Plant mitochondria also contain peroxiredoxin, thioredoxin and glutaredoxin enzyme systems capable of scavenging H_2O_2. These enzymes became a topic of great interest over the recent years (for a review see Navrot *et al*. 2007, Schwarzländer and Finkemeier 2013, Belin *et al*. 2014). In *A. thaliana* cell cultures, Cd application increased the mitochondrial peroxiredoxin protein content (Table 1.1) (Sarry *et al*. 2006). Finkemeier *et al*. (2005) demonstrated a principal role for the mitochondrial peroxiredoxin isoform F (PrxII F) in antioxidant defence and potential redox signalling in plant cells using knockout (KO)-*At*PrxII F *Arabidopsis* seedlings. Under $CdCl_2$ exposure and after SHAM-administration, the root growth of KO seedlings was more compromised as compared to wild-type seedlings, thereby signifying the involvement of this mitochondrial peroxiredoxin in Cd detoxification (Finkemeier *et al*. 2005). Gelhaye *et al*. (2004) have demonstrated the presence of a mitochondrial thioredoxin isoform in plant mitochondria capable of AOX regulation. This isoform might therefore be an interesting candidate to modulate Cd-induced mitochondrial responses via AOX. Lastly, Smiri *et al*. (2010) observed a Cd-evoked decrease in glutaredoxin activity measured in mitochondria extracts from germinating pea seeds. Although these results point towards the possible involvement of peroxiredoxin, thioredoxin and glutaredoxin enzyme systems in mitochondrial stress responses, the exact mechanisms under Cd exposure need to be resolved.

1.4. Cellular Acclimation Mechanisms Related to Mitochondria

Changes in mitochondrial electron transport and/or ROS production can have consequences for all other organelles in the plant cell. Indeed, plant mitochondria are centrally positioned in the cellular carbon and nitrogen metabolism via the TCA cycle and their role in photorespiration (Sweetlove *et al*. 2007). Dutilleul *et al*. (2003) have demonstrated this using a *Nicotiana sylvestris*

mutant (CMSII) lacking functional complex I. This induces signalling throughout the cell to reset its antioxidative capacity completely, thereby coping with the loss of a major NADH sink and enhancing resistance to ozone and *Tobacco mosaic virus* (Dutilleul *et al*. 2003). Schwarzländer *et al*. (2009) studied the importance of mitochondria in oxidative stress and redox signalling by assessing the *in vivo* oxidation state of a redox-sensitive GFP targeted to *Arabidopsis* mitochondria. They demonstrated that mitochondria are highly sensitive to redox perturbation evoked by Cd, with their redox state recovering slower from an oxidative insult as compared to the cytosol or chloroplasts (Schwarzländer *et al*. 2009). In addition, the mitochondrial ETC is also required to process excess reductants originating from photosynthetic light reactions (Araújo *et al*. 2014). Overall, this suggests that mitochondria play a central role in perception as well as response signalling (Sweetlove *et al*. 2007, Schwarzländer and Finkemeier 2013) during the oxidative challenge in Cd-exposed plants. However, next to Cd-induced ROS production and the thereby imposed (cellular) oxidative challenge, a direct link between Cd and plant mitochondria is mediated by organic acids produced in the mitochondrial matrix (Plaxton and Podestá 2006). These may be directly involved in the acclimation of plant cells to enhanced Cd concentrations and will be discussed in the light of mitochondrial alternative respiration (section 1.4.4.).

1.4.1. Mitochondrial ROS-induced damage

Once ROS are formed in mitochondria of Cd-stressed plants, they can either diffuse out of the mitochondria to mediate signalling functions or induce protein, lipid and DNA damage in the organelle itself (Rhoads *et al*. 2006). To detoxify lipid peroxidation products, plant mitochondria contain amongst other mechanisms a glutathione-S-transferase (GST) that was strongly increased in Cd-exposed plant cells (Sarry *et al*. 2006). Furthermore, plant mitochondrial proteins are susceptible to metal-catalysed oxidation, leading to the irreversible formation of reactive carbonyl groups on amino acid side chains and hence reduced protein function (Tan *et al*. 2010). Substantial evidence of both TCA cycle and photorespiration pathway proteins as important targets for ROS or lipid peroxidation products produced under environmental stresses such as drought, high light and metal exposure was summarised by Taylor *et al*. (2004).

Bartoli *et al*. (2004) have demonstrated a higher carbonyl accumulation in wheat mitochondrial proteins as compared to other ROS producing organelles such as chloroplasts and peroxisomes during well-irrigated and drought stress conditions. Mitochondrial protein carbonylation is rather selective, with not all proteins being evenly susceptible to this process. This was demonstrated by Kristensen *et al*. (2004), who have shown distinct subpopulations of the mitochondrial matrix proteome to be carbonylated after Cu and H_2O_2 treatment.

1.4.2. The role of plant mitochondria in Cd-induced programmed cell death

Although enhanced mitochondrial ROS levels may serve as monitors and signal the extent of environmental stress throughout plant cells, they may also lead to oxidative damage and PCD when mitochondrial and/or cellular antioxidative defence and repair systems are overwhelmed. Programmed cell death is an active and genetically controlled process essential for growth and development, as well as for adaptation to altered environmental conditions (Gadjev *et al*. 2008). In animals, the essential role of mitochondria in the signalling pathway transducing specific signals into the execution of cell death is widely accepted (Green and Reed 1998). A similar function was suggested for plant mitochondria, with ROS and NO as interacting signals modulating plant PCD (de Pinto *et al*. 2012, Wang *et al*. 2013). Mitochondrial $O_2^{\bullet-}$ production – rather than extracellular H_2O_2 indirectly derived from NADPH oxidases – was shown to be a key event in Cd-induced cell death in tobacco cells (Garnier *et al*. 2006). In addition, NO and ROS may be co-involved in Cd-mediated PCD as shown by De Michele *et al.* (2009) and Arasimowicz-Jelonek *et al.* (2012). Recently, Liu *et al*. (2014) demonstrated the involvement of AOX in mitochondria-dependent PCD in aluminium (Al)-exposed *A. thaliana*. Overexpression of *AOX1a*, the most dominant AOX isoform, maintained mitochondrial function and promoted expression of protective functional genes. Finally, Al-induced PCD was alleviated (Liu *et al*. 2014), which further supports the possible function of AOX as a mitochondrial "survival" protein as suggested by Robson and Vanlerberghe (2002). It is hypothesised that the survival function of AOX is based on its ability to continuously suppress mitochondrial ROS generation. This further prevents oxidative damage that could otherwise evoke disturbed gene expression and

favour PCD. In addition, the maintenance of respiration in stressful conditions by the alternative route also contributes to the hypothesis of AOX as a survival protein (Robson and Vanlerberghe 2002). Although our insights are currently increasing (Vanlerberghe 2013), more research is required to fully unravel the importance of plant mitochondria and the specific mediators involved during Cd-induced PCD.

1.4.3. Mitochondrial retrograde signalling and the alternative oxidase

An altered organellar redox state generates signals that are transmitted to the nucleus in a process called retrograde signalling. This process occurs between mitochondria, chloroplasts and the nucleus and can be mediated by ROS or oxidative stress-induced secondary signals. Recently, Suzuki *et al*. (2012) reviewed the intense relationship between mitochondria and chloroplasts in stress-induced redox signalling throughout the cell. Galvez-Valdivieso and Mullineaux (2010) summarised the involvement of ROS in chloroplastic retrograde signalling, for which more data are available as compared to mitochondria (Pfannschmidt 2010).

Due to their ability to signal across organellar membranes, ROS are considered as key components transducing mitochondrial retrograde signals (Rhoads *et al*. 2006). The dynamics and specificity of ROS-induced signalling are still questioned, but were recently reviewed by Mittler *et al*. (2011). They suggest ROS signalling to be a dynamic process occurring within cells between different organelles, as well as over long distances between different cells. Recently, Sewelam *et al*. (2014) have provided evidence for a divergent modulation of the plant transcriptome by chloroplast- or peroxisome-derived H_2O_2.

In addition, ROS-induced oxidative damage to mitochondrial components may produce secondary signals. Møller and Kristensen (2004) reviewed the potential of ROS-mediated protein oxidation in plant mitochondria as a stress indicator. Oxidatively damaged proteins such as those functioning in the TCA cycle and antioxidative defence can either be degraded by proteases or serve as an alarm signal to initiate plant responses at the cellular level (Møller and Kristensen 2004, Møller and Sweetlove 2010).

The most intensively studied model for retrograde signalling between the mitochondrion and nucleus resulting in acclimation to stress conditions is AOX.

This enzyme could play a pivotal role during Cd-induced signalling in plant mitochondria since AOX transcription, protein content and/or activity/capacity are commonly increased upon metal exposure (reviewed by Keunen *et al*. 2011). To date, several proteins are demonstrated to be involved in plant mitochondrial retrograde signalling using *AOX1a* as a marker: the transcription factor ABSCISIC ACID INSENSTIVE4 (ABI4, Giraud *et al*. 2009), CYCLIN-DEPENDENT KINASE E1 (Ng *et al*. 2013a), the transcription factor WRKY40 (Van Aken *et al*. 2013) and the membrane-bound NAC transcription factors ANAC013 (De Clercq *et al*. 2013) and ANAC017 (Ng *et al*. 2013b). Recently, a functional antagonistic relationship between auxin and mitochondrial retrograde signalling has been shown to regulate *AOX1a* expression in *Arabidopsis* (Ivanova *et al*. 2014). Vanlerberghe *et al*. (2009) reviewed the postulated metabolic and physiological roles of the alternative respiratory pathway, with AOX possibly maintaining a homeostatic mitochondrial signal during stress conditions. Interestingly, the AsA biosynthesis capacity increased in isolated mitochondria of *A. thaliana* plants overexpressing AOX (Bartoli *et al*. 2006), suggesting a link between this energy-dissipating enzyme and mitochondrial and cellular antioxidative metabolism.
Although more pieces of the puzzle are currently starting to come together (Vanlerberghe 2013, Ng *et al*. 2014), further research using transgenic plants will contribute to our insights into the role of AOX in metal/Cd-induced (retrograde) signalling. However, other proteins and/or metabolites should also be studied to fully unravel the mechanisms involved in the acclimation of plants growing on metal-contaminated soils.

1.4.4. Metal tolerance mediated by mitochondrial organic acids

In stress conditions compromising the phosphorylating cytochrome respiratory pathway, such as Cd exposure, plant mitochondria use their alternative respiratory pathway to maintain the electron flux to O_2. As discussed earlier, this may reduce mitochondrial ROS production by electron leakage from the system, thereby decreasing oxidative stress at organellar and possibly cellular levels. In addition, the sustained electron flux allows a continuously operating glycolysis and TCA cycle, thereby ensuring a great metabolic flexibility under stress conditions (Plaxton and Podestá 2006). This provides an alternative for the non-phosphorylating respiratory bypasses to ameliorate Cd stress – next to their

proposed role in modulating ROS production. Indeed, an increased TCA cycle flux results in the production of organic acids in the mitochondrial matrix. These metabolites form a direct link between plant mitochondria and acclimation to metal stress as they are associated with metal hyperaccumulation and tolerance in several plant species. Citrate, malate and oxalate have been suggested as key cellular ligands for Cd, Ni and Zn, mediating metal transport through the xylem and vacuolar sequestration of metal-ligand complexes (for reviews see Rauser 1999, Plaxton and Podestá 2006, Haydon and Cobbett 2007 and references therein). In *Lycopersicon esulentum*, Cd-induced secretion of oxalate from root apices was found to be associated with Cd resistance (Zhu *et al*. 2011). Interestingly, Gray *et al*. (2004) have demonstrated an increased *AOX1* transcript level in tobacco cells incubated with organic acids such as citrate, malate and 2-oxoglutarate in a physiologically relevant concentration range. The authors postulated a plant mitochondrial retrograde signalling pathway for the regulation of AOX gene expression based on TCA cycle intermediates, which could function concomitantly with ROS signalling to the nucleus (Gray *et al*. 2004). However, later studies in rice and *Arabidopsis* did not support the role of citrate as retrograde signalling molecule for *AOX1a* induction (reviewed by Ng *et al*. 2014).

1.5. Concluding Remarks

Although the overall phytotoxic response to Cd exposure is largely understood, the underlying molecular mechanisms are not fully elucidated yet. Nevertheless, this fundamental knowledge is highly useful to develop or adjust strategies to cope with metal-polluted soils, for example in search of potential biomarkers. Due to their central position in the cellular metabolism, the close relationship with chloroplasts and the integration of redox signals, plant mitochondria are suggested to be main players in Cd-induced cellular responses. In addition to being a target of cytosolic ROS, they also represent an important source of ROS production in Cd stress conditions. Depending on the intensity of the stressor, ROS are able to induce mitochondrial damage and/or signalling outside the mitochondria. The involvement of (mitochondrial) ROS in Cd-induced PCD has been confirmed in several studies. Functioning as both target and regulator of stress responses in plants, AOX is of major importance in the mitochondrial

metabolism. Due to its ability to reduce mitochondrial ROS production and modulate PCD and TCA cycle activity, AOX is suggested to play a key role in Cd-induced responses in plant mitochondria (Figure 1.6). Further research is needed to explore the role of this and other mitochondrial proteins in PCD and/or signalling-induced acclimation in more detail. Unravelling the involvement of mitochondria in the Cd-induced oxidative challenge will ultimately contribute to the development and/or selection of crops with enhanced yield under suboptimal conditions such as Cd exposure, thereby improving and safeguarding the role of plants in a sustainable future.

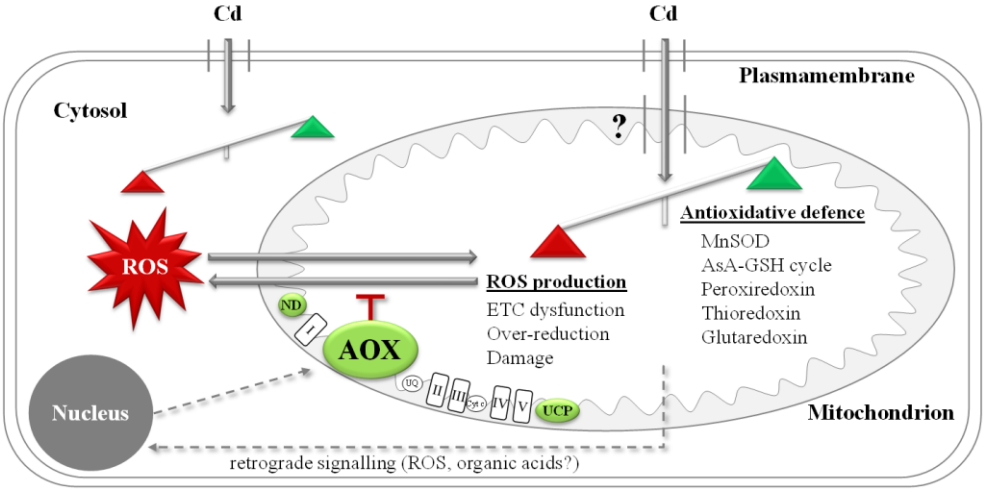


Figure 1.6. *Schematic overview of Cd-induced responses in plant cells focussing on mitochondrial effects. Metal exposure has shown to cause mitochondrial electron transport chain (ETC) dysfunction and over-reduction, thereby increasing mitochondrial ROS production. However, more research is needed to determine whether this is the direct consequence of Cd entering the mitochondria, since cytosolic ROS production cannot be excluded in the light of metal stress and can influence mitochondrial responses. As they are able to cross cellular membranes, ROS serve signalling functions outside the mitochondria (dashed line) and can induce retrograde signalling to the nucleus, which could also be regulated via organic acids. As AOX is able to reduce mitochondrial ROS production and can modulate programmed cell death (PCD) and tricarboxylic acid (TCA) cycle activity, this enzyme is suggested to play a key role in Cd-induced responses in plant mitochondria.*

REFERENCES

Adriano, D.C. (2001) Cadmium, in: Adriano, D.C. (Ed.), Trace elements in terrestrial environments. Biogeochemistry, bioavailability, and risks of metals. Springer-Verlag, New York, Berlin, Heidelberg, pp. 263–314.

Alscher, R.G., Erturk, N. and Heath, L.S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot*, **53**, 1331–1341.

Amirsadeghi, S., Robson, C.A. and Vanlerberghe, G.C. (2007) The role of the mitochondrion in plant responses to biotic stress. *Physiol Plant*, **129**, 253–266.

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J. and Gwóźdź, E.A. (2011) The message of nitric oxide in cadmium challenged plants. *Plant Sci*, **181**, 612–620.

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., Deckert, J., Rucínska-Sobkowiak, R., Gzyl, J., Pawlak-Sprada, S., Abramowski, D., Jelonek, T. and Gwóźdź, E.A. (2012) Nitric oxide implication in cadmium-induced programmed cell death in roots and signaling response of yellow lupine plants. *Plant Physiol Biochem*, **58**, 124–134.

Araújo, W.L., Nunes-Nesi, A. and Fernie, A.R. (2014) On the role of plant mitochondrial metabolism and its impact on photosynthesis in both optimal and sub-optimal growth conditions. *Photosynth Res*, **119**, 141–156.

Asada, K. (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol*, **141**, 391–396.

ATSDR (2008) Agency for Toxic Substances and Disease Registry, U.S. Toxicological Profile for Cadmium. Department of Health and Human Services, Public Health Service, Atlanta, GA. (http://www.atsdr.cdc.gov/toxprofiles/tp5.pdf)

Bartoli, C.G., Pastori, G.M. and Foyer, C.H. (2000) Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol*, **123**, 335–343.

Bartoli, C.G., Gómez, F., Martínez, D.E. and Guiamet, J.J. (2004) Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). *J Exp Bot*, **55**, 1663–1669.

Bartoli, C.G., Yu, J., Gómez, F., Fernández, L., McIntosh, L. and Foyer, C.H. (2006) Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in *Arabidopsis thaliana* leaves. *J Exp Bot*, **57**, 1621–1631.

Belin, C., Bashandy, T., Cela, J., Delorme-Hinoux, V., Riondet, C. and Reichheld, J.P. (2014) A comprehensive study of thiol reduction gene expression under stress conditions in *Arabidopsis thaliana*. *Plant Cell Environ*, doi: 10.1111/pce.12276.

Bi, Y.H., Chen, W.L., Zhang, W.N., Zhou, Q., Yun L.J. and Xing, D. (2009) Production of reactive oxygen species, impairment of photosynthetic function and dynamic changes in mitochondria are early events in cadmium-induced cell death in *Arabidopsis thaliana*. *Biol Cell*, **101**, 629–643.

Blokhina, O. and Fagerstedt, K.V. (2010) Reactive oxygen species and nitric oxide in plant mitochondria: origin and redundant regulatory systems. *Physiol Plant*, **138**, 447–462.

Brandalise, M., de Godoy Maia, I., Borecký, J., Vercesi, A.E. and Arruda, P. (2003) ZmPUMP encodes a maize mitochondrial uncoupling protein that is induced by oxidative stress. *Plant Sci*, **165**, 329–335.

Castro-Guerrero, N.A., Rodríguez-Zavala, J.S., Marín-Hernández, A., Rodríguez-Enríquez, S. and Moreno-Sánchez, R. (2008) Enhanced alternative oxidase and antioxidant enzymes under Cd^{2+} stress in *Euglena*. *J Bioenerg Biomembr*, **40**, 227–235.

Chary, N.S., Kamala, C.T. and Raj, D.S.S. (2008) Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. *Ecotoxicol Environ Saf*, **69**, 513–524.

Chew, O., Whelan, J. and Millar, A.H. (2003) Molecular definition of the ascorbate-glutathione cycle in *Arabidopsis* mitochondria reveals dual targeting of antioxidant defenses in plants. *J Biol Chem*, **278**, 46869–46877.

Clemens, S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie*, **88**, 1707–1719.

Clemens, S., Aarts, M.G.M., Thomine, S. and Verbruggen, N. (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci*, **18**, 92–99.

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A. and Whelan, J. (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol Biol*, **58**, 193–212.

Considine, M.J., Goodman, M., Echtay, K.S., Laloi, M., Whelan, J., Brand, M.D. and Sweetlove, L.J. (2003) Superoxide stimulates a proton leak in potato mitochondria that is related to the activity of uncoupling protein. *J Biol Chem*, **278**, 22298–22302.

Cuypers, A., Smeets, K. and Vangronsveld, J. (2009) Heavy metal stress in plants, in: Hirt, H. (Ed.), Plant stress biology. From genomics to systems biology. Wiley-VCH Verlagsgesellschaft GmbH & Co. KGaA., Weinheim, pp. 161–178.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J. and Smeets, K. (2010) Cadmium stress: an oxidative challenge. *Biometals*, **23**, 927–940.

Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J. and Vangronsveld, J. (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J Plant Physiol*, **168**, 309–316.

Cuypers, A., Keunen, E., Bohler, S., Jozefczak, M., Opdenakker, K., Gielen, H., Vercampt, H., Bielen, A., Schellingen, K., Vangronsveld, J. and Remans, T. (2012) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling, in: Gupta, D.K., Sandalio, L.M. (Eds.), Metal toxicity in plants: Perception, signaling and remediation. Springer-Verlag GmbH, Berlin, Heidelberg, pp. 65–90.

Cvetkovska, M. and Vanlerberghe, G.C. (2012) Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol*, **195**, 32–39.

DalCorso, G., Farinati, S., Maistri, S. and Furini, A. (2008) How plants cope with cadmium: Staking all on metabolism and gene expression. *J Integr Plant Biol*, **50**, 1268–1280.

Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D. and Van Breusegem, F. (2000) Dual action of the active oxygen species during plant stress responses. *Cell Mol Life Sci*, **57**, 779–795.

De Clercq, I., Vermeirssen, V., Van Aken, O., Vandepoele, K., Murcha, M.W., Law, S.R., Inzé, A., Ng, S., Ivanova, A., Rombaut, D., van de Cotte, B., Jaspers, P., Van de Peer, Y., Kangasjärvi, J., Whelan, J. and Van Breusegem, F. (2013) The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in *Arabidopsis*. *Plant Cell*, **25**, 3472–3490.

De Gara, L., Locato, V., Dipierro, S. and de Pinto, M.C. (2010) Redox homeostasis in plants. The challenge of living with endogenous oxygen production. *Respir Physiol Neurobiol*, **173**, S13–S19.

De Michele, R., Vurro, E., Rigo, C., Costa, A., Elviri, L., Di Valentin, M., Careri, M., Zottini, M., Sanità di Toppi, L. and Lo Schiavo, F. (2009) Nitric oxide is involved in cadmium-induced programmed cell death in Arabidopsis suspension cultures. *Plant Physiol*, **150**, 217–228.

de Pinto, M.C., Locato, V. and De Gara, L. (2012) Redox regulation in plant programmed cell death. *Plant Cell Environ*, **35**, 234–244.

Desikan, R., A.-H.-Mackerness, S., Hancock, J.T. and Neill, S.J. (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. *Plant Physiol*, **127**, 159–172.

Dutilleul, C., Garmier, M., Noctor, G., Mathieu, C., Chétrit, P., Foyer, C.H. and De Paepe, R. (2003) Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. *Plant Cell*, **15**, 1212–1226.

Faller, P., Kienzler, K. and Krieger-Liszkay, A. (2005) Mechanism of Cd^{2+} toxicity: Cd^{2+} inhibits photoactivation of photosystem II by competitive binding to the essential Ca^{2+} site. *Biochim Biophys Acta*, **1706**, 158–164.

Fernie, A.R., Carrari, F. and Sweetlove, L.J. (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Curr Opin Plant Biol*, **7**, 254–261.

Finkemeier, I., Goodman, M., Lamkemeyer, P., Kandlbinder, A., Sweetlove, L.J. and Dietz, K.J. (2005) The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of *Arabidopsis thaliana* under stress. *J Biol Chem*, **280**, 12168–12180.

Gadjev, I., Stone, J.M. and Gechev, T.S. (2008) Programmed cell death in plants: New insights into redox regulation and the role of hydrogen peroxide. *Int Rev Cell Mol Biol*, **270**, 87–144.

Galvez-Valdivieso, G. and Mullineaux, P.M. (2010) The role of reactive oxygen species in signalling from chloroplasts to the nucleus. *Physiol Plant*, **138**, 430–439.

Garmash, E.V. and Golovko, T.K. (2009) Effect of cadmium on growth and respiration of barley plants grown under two temperature regimes. *Russ J Plant Physiol*, **56**, 343–347.

Garnier, L., Simon-Plas, F., Thuleau, P., Agnel, J.P., Blein, J.P., Ranjeva, R. and Montillet, J.L. (2006) Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant Cell Environ*, **29**, 1956–1969.

Gechev, T.S., Van Breusegem, F., Stone, J.M., Denev, I. and Laloi, C. (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays*, **28**, 1091–1101.

Gelhaye, E., Rouhier, N., Gérard, J., Jolivet, Y., Gualberto, J., Navrot, N., Ohlsson, P.I., Wingsle, G., Hirasawa, M., Knaff, D.B., Wang, H., Dizengremel, P. Meyer, Y. and Jacquot, J.P. (2004) A specific form of thioredoxin *h* occurs in plant mitochondria and regulates the alternative oxidase. *Proc Natl Acad Sci USA*, **101**, 14545–14550.

Ghezzi, P. and Bonetto, V. (2003) Redox proteomics: Identification of oxidatively modified proteins. *Proteomics*, **3**, 1145–1153.

Gill, S.S. and Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem*, **48**, 909–930.

Giraud, E., Van Aken, O., Ho, L.H.M. and Whelan, J. (2009) The transcription factor ABI4 is a regulator of mitochondrial retrograde expression of *ALTERNATIVE OXIDASE1a*. *Plant Physiol*, **150**, 1286–1296.

Gray, G.R., Maxwell, D.P., Villarimo, A.R. and McIntosh, L. (2004) Mitochondria/nuclear signaling of alternative oxidase gene expression occurs through distinct pathways involving organic acids and reactive oxygen species. *Plant Cell Rep*, **23**, 497–503.

Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309–1312.

Groß, F., Durner, J. and Gaupels, F. (2013) Nitric oxide, antioxidants and prooxidants in plant defence responses. *Front Plant Sci*, **4**, 419.

Halliwell, B. (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol*, **141**, 312–322.

Haydon, M.J. and Cobbett, C.S. (2007) Transporters of ligands for essential metal ions in plants. *New Phytol*, **174**, 499–506.

Heyno, E., Klose, C. and Krieger-Liszkay, A. (2008) Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. *New Phytol*, **179**, 687–699.

Igamberdiev, A.U., Ratcliffe, R.G. and Gupta, K.J. (2014) Plant mitochondria: Source and target for nitric oxide. *Mitochondrion*, doi: 10.1016/j.mito.2014.02.003.

Ivanova, A., Law, S.R., Narsai, R., Duncan, O., Lee, J.H., Zhang, B., Van Aken, O., Radomiljac, J.D., van der Merwe, M., Yi, K. and Whelan, J. (2014) A functional antagonistic relationship between auxin and mitochondrial retrograde signaling regulates ALTERNATIVE OXIDASE1a expression in Arabidopsis thaliana. *Plant Physiol*, **165**, 1233–1254.

Jacques, S., Ghesquière, B., Van Breusegem, F. and Gevaert, K. (2013) Plant proteins under oxidative attack. *Proteomics,* **13**, 932–940.

Järup, L. and Åkesson, A. (2009) Current status of cadmium as an environmental health problem. *Toxicol Appl Pharmacol*, **238**, 201–208.

Jiménez, A., Hernández, J.A., del Río, L.A. and Sevilla, F. (1997) Evidence for the presence of the ascorbate glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol*, **114**, 275–284.

Jozefczak, M., Keunen, E., Schat, H., Bliek, M., Hernández, L.E., Carleer, R., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2014) Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. *Plant Physiol Biochem*, **83**, 1–9.

Keunen, E., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2011) Metal-induced oxidative stress and plant mitochondria. *Int J Mol Sci*, **12**, 6894–6918.

Keunen, E., Remans, T., Opdenakker, K., Jozefczak, M., Gielen, H., Guisez, Y., Vangronsveld, J. and Cuypers, A. (2013) A mutant of the *Arabidopsis thaliana* LIPOXYGENASE1 gene shows altered signalling and oxidative stress related responses after cadmium exposure. *Plant Physiol Biochem*, **63**, 272–280.

Khan, M.A., Castro-Guerrero, N. and Mendoza-Cozatl, D.G. (2014) Moving towards a precise nutrition: preferential loading of seeds with essential nutrients over non-essential toxic elements. *Front Plant Sci*, **5**, 51.

Kristensen, B.K., Askerlund, P., Bykova, N.V., Egsgaard, H. and Møller, I.M. (2004) Identification of oxidised proteins in the matrix of rice leaf mitochondria by immunoprecipitation and two-dimensional liquid chromatography-tandem mass spectrometry. *Phytochemistry*, **65**, 1839–1851.

Kučera, T., Horáková, H. and Šonská, A. (2008) Toxic metal ions in photoautotrophic organisms. *Photosynthetica*, **46**, 481–489.

Laloi, M., Klein, M., Riesmeier, J.W., Müller-Röber, B., Fleury, C., Bouillaud, F. and Ricquier, D. (1997) A plant cold-induced uncoupling protein. *Nature*, **389**, 135–136.

Lane, T.W. and Morel, F.M. (2000) A biological function for cadmium in marine diatoms. *Proc Natl Acad Sci USA*, **97**, 4627–4631.

Li, C.R., Liang, D.D., Li, J., Duan, Y.B., Li, H., Yang, Y.C., Qin, R.Y., Li, L., Wei, P.C. and Yang, J.B. (2013) Unravelling mitochondrial retrograde regulation in the abiotic stress induction of rice *ALTERNATIVE OXIDASE 1* genes. *Plant Cell Environ*, **36**, 775–788.

Liu, J., Li, Z., Wang, Y.Q. and Xing, D. (2014) Overexpression of ALTERNATIVE OXIDASE1a alleviates mitochondria-dependent programmed cell death induced by aluminium phytotoxicity in *Arabidopsis*. *J Exp Bot*, **65**, 4465–4478.

Maxwell, D.P., Wang, Y. and McIntosh, L. (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc Natl Acad Sci USA*, **96**, 8271–8276.

Mendoza-Cózatl, D.G., Jobe, T.O., Hauser, F. and Schroeder, J.I. (2011) Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. *Curr Opin Plant Biol*, **14**, 554–562.

Meyer, C.L. and Verbruggen, N. (2012) The use of the model species *Arabidopsis halleri* towards phytoextraction of cadmium polluted soils. *New Biotechnol*, **30**, 9–14.

Millar, H., Considine, M.J., Day, D.A. and Whelan, J. (2001) Unraveling the role of mitochondria during oxidative stress in plants. *IUBMB Life*, **51**, 201–205.

Millar, A.H., Whelan, J., Soole, K.L. and Day, D.A. (2011) Organization and regulation of mitochondrial respiration in plants. *Annu Rev Plant Biol*, **62**, 79–104.

Miller, G., Shulaev, V. and Mittler, R. (2008) Reactive oxygen signaling and abiotic stress. *Physiol Plant*, **133**, 481–489.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S. and Mittler, R. (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ*, **33**, 453–467.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci*, **9**, 490–498.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V.B., Vandepoele, K., Gollery, M., Shulaev, V. and Van Breusegem, F. (2011) ROS signaling: the new wave? *Trends Plant Sci*, **6**, 300–309.

Møller, I.M. (2001) Plant mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annu Rev Plant Physiol Plant Mol Biol*, **52**, 561–591.

Møller, I.M. and Kristensen, B.K. (2004) Protein oxidation in plant mitochondria as a stress indicator. *Photoch Photobio Sci*, **3**, 730–735.

Møller, I.M., Jensen, P.E. and Hansson, A. (2007) Oxidative modifications to cellular components in plants. *Annu Rev Plant Biol*, **58**, 459–481.

Møller, I.M. and Sweetlove, L.J. (2010) ROS signalling - specificity is required. *Trends Plant Sci*, **15**, 370–374.

Nair, A.R., DeGheselle, O., Smeets, K., Van Kerkhove, E. and Cuypers, A. (2013) Cadmium-induced pathologies: Where is the oxidative balance lost (or not)? *Int J Mol Sci*, **14**, 6116–6143.

Navrot, N., Rouhier, N., Gelhaye, E. and Jacquot, J.P. (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiol Plant*, **129**, 185–195.

Nawrot, T.S., Staessen, J.A., Roels, H.A., Munters, E., Cuypers, A., Richart, T., Ruttens, A., Smeets, K., Clijsters, H. and Vangronsveld, J. (2010) Cadmium exposure in the population: from health risks to strategies of prevention. *Biometals*, **23**, 769–782.

Ng, S., Giraud, E., Duncan, O., Law, S.R., Wang, Y., Xu, L., Narsai, R., Carrie, C., Walker, H., Day, D.A., Blanco, N.E., Strand, Å., Whelan, J. and Ivanova, A. (2013a) Cyclin-dependent kinase E1 (CDKE1) provides a cellular switch in plants between growth and stress responses. *J Biol Chem*, **288**, 3449–3459.

Ng, S., Ivanova, A., Duncan, O., Law, S.R., Van Aken, O., De Clercq, I., Wang, Y., Carrie, C., Xu, L., Kmiec, B., Walker, H., Van Breusegem, F., Whelan, J. and Giraud, E. (2013b) A membrane-bound NAC transcription factor, ANAC017, mediates mitochondrial retrograde signaling in *Arabidopsis*. *Plant Cell*, **25**, 3450–3471.

Ng, S., De Clercq, I., Van Aken, O., Law, S.R., Ivanova, A., Willems, P., Giraud, E., Van Breusegem, F. and Whelan, J. (2014) Anterograde and retrograde regulation of nuclear genes encoding mitochondrial proteins during growth, development, and stress. *Mol Plant*, **7**, 1075–1093.

Noctor, G., De Paepe, R. and Foyer, C.H. (2007) Mitochondrial redox biology and homeostasis in plants. *Trends Plant Sci*, **12**, 125–134.

Nogueira, F.T.S., Sassaki, F.T. and Maia, I.G. (2011) *Arabidopsis thaliana* Uncoupling Proteins (AtUCPs): insights into gene expression during development and stress response and epigenetic regulation. *J Bioenerg Biomembr*, **43**, 71–79.

Nriagu, J.O. and Pacyna, J.M. (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature*, **333**, 134–139.

Ozawa, K., Murayama, S., Kobayashi-Uehara, A. and Handa, H. (2006) Overexpression of wheat mitochondrial uncoupling protein in rice plants confers tolerances to oxidative stress promoted by exogenous hydrogen peroxide and low temperature. *Mol Breed*, **18**, 51–56.

Pastore, D., Trono, D., Laus, M.N., Di Fonzo, N. and Flagella, Z. (2007) Possible plant mitochondria involvement in cell adaptation to drought stress. A case study: durum wheat mitochondria. *J Exp Bot*, **58**, 195–210.

Petrov, V.D. and Van Breusegem, F. (2012) Hydrogen peroxide – a central hub for information flow in plant cells. *AoB PLANTS*, pls014.

Pfannschmidt, T. (2010) Plastidial retrograde signalling - a true "plastid factor" or just metabolite signatures? *Trends Plant Sci*, **15**, 427–435.

Plaxton, W.C. and Podestá, F.E. (2006) The functional organization and control of plant respiration. *Crit Rev Plant Sci*, **25**, 159–198.

Purvis, A.C. and Shewfelt, R.L. (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiol Plant*, **88**, 712–718.

Rasmusson, A.G., Geisler, D.A. and Møller, I.M. (2008) The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. *Mitochondrion*, **8**, 47–60.

Rasmusson, A.G., Fernie, A.R. and van Dongen, J.T. (2009) Alternative oxidase: a defence against metabolic fluctuations? *Physiol Plant*, **137**, 371–382.

Rasmusson, A.G. and Møller, I.M. (2011) Mitochondrial Electron Transport and Plant Stress, in: Kempken, F. (Ed.), Plant Mitochondria. Advances in Plant Biology. Springer, New York, pp. 357–381.

Rauser, W.E. (1999) Structure and function of metal chelators produced by plants - The case for organic acids, amino acids, phytin, and metallothioneins. *Cell Biochem Biophys*, **31**, 19–48.

Reese, R.N. and Roberts, L.W. (1985) Effects of cadmium on whole cell and mitochondrial respiration in tobacco cell suspension cultures (*Nicotiana tabacum* L. var. *xanthi*). *J Plant Physiol*, **120**, 123–130.

Remans, T., Opdenakker, K., Smeets, K., Mathijsen, D., Vangronsveld, J. and Cuypers, A. (2010) Metal-specific and NADPH oxidase dependent changes in lipoxygenase and NADPH oxidase gene expression in *Arabidopsis thaliana* exposed to cadmium or excess copper. *Funct Plant Biol*, **37**, 532–544.

Rhoads, D.M., Umbach, A.L., Subbaiah, C.C. and Siedow, J.N. (2006) Mitochondrial reactive oxygen species. Contribution to oxidative stress and interorganellar signaling. *Plant Physiol*, **141**, 357–366.

Rhoads, D.M. and Subbaiah, C.C. (2007) Mitochondrial retrograde regulation in plants. *Mitochondrion*, **7**, 177–194.

Robson, C.A. and Vanlerberghe, G.C. (2002) Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. *Plant Physiol*, **129**, 1908–1920.

Rodríguez-Serrano, M., Romero-Puertas, M.C., Sparkes, I., Hawes, C., del Río, L.A. and Sandalio, L.M. (2009) Peroxisome dynamics in Arabidopsis plants under oxidative stress induced by cadmium. *Free Radical Biol Med*, **47**, 1632–1639.

Sarry, J.E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain, A., Bastien, O., Fievet, J.B., Vailhen, D., Amekraz, B., Moulin, C., Ezan, E., Garin, J. and Bourguignon, J. (2006) The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics*, **6**, 2180–2198.

Schützendübel, A. and Polle, A. (2002) Plant responses to abiotic stress: heavy-metal induced oxidative stress and protection by mycorrhization. *J Exp Bot*, **53**, 1351–1365.

Schwarzländer, M., Fricker, M.D. and Sweetlove, L.J. (2009) Monitoring the *in vivo* redox state of plant mitochondria: Effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. *Biochim Biophys Acta*, **1787**, 468–475.

Schwarzländer, M. and Finkemeier, I. (2013) Mitochondrial energy and redox signaling in plants. *Antioxid Redox Signal*, **18**, 2122–2144.

Seth, C.S., Remans, T., Keunen, E., Jozefczak, M., Gielen, H., Opdenakker, K., Weyens, N., Vangronsveld, J. and Cuypers, A. (2012) Phytoextraction of toxic metals: a central role for glutathione. *Plant Cell Environ*, **35**, 334–346.

Sewelam, N., Jaspert, N., Van der Kelen, K., Tognetti, V.B., Schmitz, J., Frerigmann, H., Stahl, E., Zeier, J., Van Breusegem, F. and Maurino, V.G. (2014) Spatial H_2O_2 signalling specificity: H_2O_2 from chloroplasts and peroxisomes modulates the plant transcriptome differentially. *Mol Plant*, **7**, 1191–1210.

Sharma, S.S. and Dietz, K.J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci*, **14**, 43–50.

Smiri, M., Chaoui, A., Rouhier, N., Kamel, C., Gelhaye, E., Jacquot, J.P. and El Ferjani, E. (2010) Cadmium induced mitochondrial redox changes in germinating pea seed. *Biometals*, **23**, 973–984.

Smith, A.M.O., Ratcliffe, R.G. and Sweetlove, L.J. (2004) Activation and function of mitochondrial uncoupling protein in plants. *J Biol Chem*, **279**, 51944–51952.

Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M.A. and Mittler, R. (2011) Respiratory burst oxidases: the engines of ROS signaling. *Curr Opin Plant Biol*, **14**, 691–699.

Suzuki, N., Koussevitzky, S., Mittler, R. and Miller, G. (2012) ROS and redox signalling in the response of plants to abiotic stress. *Plant Cell Environ*, **35**, 259–270.

Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J. and Millar, A.H. (2002) The impact of oxidative stress on *Arabidopsis* mitochondria. *Plant J*, **32**, 891–904.

Sweetlove, L.J., Lytovchenko, A., Morgan, M., Nunes-Nesi, A., Taylor, N.L., Baxter, C.J., Eickmeier, I. and Fernie, A.R. (2006) Mitochondrial uncoupling protein is required for efficient photosynthesis. *Proc Natl Acad Sci USA*, **103**, 19587–19592.

Sweetlove, L.J., Fait, A., Nunes-Nesi, A., Williams, T. and Fernie, A.R. (2007) The mitochondrion: An integration point of cellular metabolism and signalling. *Crit Rev Plant Sci*, **26**, 17–43.

Szarka, A., Horemans, N., Kovács, Z., Gróf, P., Mayer, M. and Bánhegyi, G. (2007) Dehydroascorbate reduction in plant mitochondria is coupled to the respiratory electron transfer chain. *Physiol Plant*, **129**, 225–232.

Tamas, L., Dudikova, J., Durcekova, K., Haluskova, L., Huttova, J. and Mistrik, I. (2009) Effect of cadmium and temperature on the lipoxygenase activity in barley root tip. *Protoplasma*, **235**, 17–25.

Tan, Y.F., O'Toole, N., Taylor, N.L. and Millar, A.H. (2010) Divalent metal ions in plant mitochondria and their role in interactions with proteins and oxidative stress-induced damage to respiratory function. *Plant Physiol*, **152**, 747–761.

Taylor, N.L., Day, D.A. and Millar, A.H. (2004) Targets of stress-induced oxidative damage in plant mitochondria and their impact on cell carbon/nitrogen metabolism. *J Exp Bot*, **55**, 1–10.

Van Aken, O., Giraud, E., Clifton, R. and Whelan, J. (2009) Alternative oxidase: a target and regulator of stress responses. *Physiol Plant*, **137**, 354–361.

Van Aken, O., Zhang, B., Law, S., Narsai, R. and Whelan, J. (2013) AtWRKY40 and AtWRKY63 modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins. *Plant Physiol*, **162**, 254–271.

Van Belleghem, F., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, J., d'Haen, J. and Valcke R. (2007) Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytol*, **173**, 495–508.

van Dongen, J.T., Gupta, K.J., Ramírez-Aguilar, S.J., Araújo, W.L., Nunes-Nesi, A. and Fernie, A.R. (2011) Regulation of respiration in plants: A role for alternative metabolic pathways. *J Plant Physiol*, **168**, 1434–1443.

Vangronsveld, J., Van Assche, F. and Clijsters, H. (1995) Reclamation of a bare industrial area contaminated by non-ferrous metals: *In situ* metal immobilization and revegetation. *Environ Pollut*, **87**, 51–59.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D. and Mench, M. (2009) Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res*, **16**, 765–794.

Vanlerberghe, G.C., Cvetkovska, M. and Wang, J. (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? *Physiol Plant*, **137**, 392–406.

Vanlerberghe, G.C. (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci*, **14**, 6805–6847.

Verbruggen, N., Hermans, C. and Schat, H. (2009) Mechanisms to cope with arsenic or cadmium excess in plants. *Curr Opin Plant Biol*, **12**, 364–372.

Verbruggen, N., Juraniec, M., Baliardini, C. and Meyer, C.L. (2013) Tolerance to cadmium in plants: the special case of hyperaccumulators. *Biometals*, **26**, 633–638.

Vercesi, A.E., Borecký, J., de Godoy Maia, I., Arruda, P., Cuccovia, I.M. and Chaimovich, H. (2006) Plant uncoupling mitochondrial proteins. *Annu Rev Plant Biol*, **57**, 383–404.

Wang, Y., Loake, G.J. and Chu, C. (2013) Cross-talk of nitric oxide and reactive oxygen species in plant programmed cell death. *Front Plant Sci*, **4**, 314.

WHO (2010) World Health Organization. Exposure to cadmium: a major public health concern. Public Health and Environment, Geneva, Switzerland.

Wong, C.K.E. and Cobbett, C.S. (2009) HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*. *New Phytol*, **181**, 71–78.

Wong, H.L., Pinontoan, R., Hayashi, K., Tabata, R., Yaeno, T., Hasegawa, K., Kojima, C., Yoshioka, H., Iba, K., Kawasaki, T. and Shimamoto, K. (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. *Plant Cell*, **19**, 4022–4034.

Yamamoto, Y., Kobayashi, Y., Devi, S.R., Rikiishi, S. and Matsumoto, H. (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. *Plant Physiol*, **128**, 63–72.

Yin, L., Mano, J., Wang, S., Tsuji, W. and Tanaka, K. (2010) The involvement of lipid peroxide-derived aldehydes in aluminum toxicity of tobacco roots. *Plant Physiol*, **152**, 1406–1417.

Zhao, Z.Q., Cai, Y.L., Zhu, Y.G. and Kneer, R. (2005) Cadmium-induced oxidative stress and protection by L-galactono-1,4-lactone in winter wheat (*Triticum aestivum* L.). *J Plant Nutr Soil Sci*, **168**, 759–763.

Zhu, X.F., Zheng, C., Hu, Y.T., Jiang, T., Liu, Y., Dong, N.Y., Yang, J.L. and Zheng, S.J. (2011) Cadmium-induced oxalate secretion from root apex is associated with cadmium exclusion and resistance in *Lycopersicon esulentum*. *Plant Cell Environ*, **34**, 1055–1064.

CHAPTER 2
Objectives

Due to natural but mainly anthropogenic sources, soil metal pollution is a challenging problem that several regions worldwide – including the Belgian Campine region – are confronted with. Even the presence of trace amounts of the non-essential toxic element cadmium (Cd) can be a threat for the environment and human health as it readily accumulates into the food chain (Clemens *et al*. 2013). Cadmium disturbs several physiological processes in plants such as photosynthesis and respiration. In addition, its phytotoxicity is highly associated with an increased production of reactive oxygen species (ROS), thereby imposing a cellular oxidative challenge. On the one hand, ROS are able to oxidatively damage plant DNA, proteins and lipids. However, they also act as signalling components potentially involved in plant acclimation to Cd stress (Cuypers *et al*. 2012).

Subcellularly, plant mitochondria are central hubs in redox homeostasis and signalling as they constantly produce ROS as by-products at the level of the electron transport chain (ETC). This ETC is rapidly targeted by Cd, thereby increasing mitochondrial ROS production. In this way, plant mitochondria could be involved in perception as well as response signalling during Cd exposure (Keunen *et al*. 2011 and references therein). In the plant mitochondrial ETC, two terminal oxidases are present. In addition to cytochrome c oxidase, an alternative oxidase (AOX) couples the oxidation of ubiquinol to the reduction of O_2 to H_2O. This enzyme is commonly considered as a crucial mediator of plant stress responses (Vanlerberghe 2013). Recently, direct *in planta* evidence has indicated that leaves of AOX-suppressed tobacco plants show increased mitochondrial superoxide ($O_2^{\bullet -}$) levels (Cvetkovska and Vanlerberghe 2012). Thus, AOX could be involved in ROS-mediated signalling starting from the mitochondrion. In addition, AOX impacts the cellular energy status. As it does not pump protons across the inner membrane and bypasses proton-pumping complexes III and IV, AOX activity drastically reduces respiratory energy (ATP) yield. The presence of AOX allows a flexible degree of coupling between carbon metabolism (tricarboxylic acid (TCA) cycle), ETC activity and ATP synthesis, thereby contributing to metabolic homeostasis (Vanlerberghe 2013).

In this study, it is therefore hypothesised that the mitochondrial AOX is of central importance in the response of plants to sublethal Cd exposure in relation to the oxidative challenge. The primary goal is to extend our knowledge on (1) the oxidative challenge induced by Cd and (2) how this is related to mitochondria and AOX in particular in the model organism *Arabidopsis thaliana* during environmentally realistic exposure conditions.

To this end, three research questions have been put forward in the outline of this thesis:

1. Which Cd concentrations are suitable to study stress responses in a sublethal yet toxic range in *A. thaliana* plants?

A first challenge was to determine those Cd concentrations suitable *i.e.* environmentally realistic, sublethal but toxic to investigate Cd-induced damage versus signalling responses in *A. thaliana* plants grown in hydroponics. Exposing *A. thaliana* to a range of environmentally realistic Cd concentrations (5, 10 or 20 µM $CdSO_4$) during 24 h showed that the highest concentration was already too toxic to include in further studies (Smeets *et al*. 2008). Therefore, it was studied whether chronic exposure to 5 or 10 µM Cd affects the ability of *A. thaliana* plants to complete their life cycle in a validated hydroponic setup **(Chapter 3)**. This experiment ensured the non-lethality of the selected Cd concentrations to study moderate toxicity within a short-term exposure period up to 72 h.

2. Is the mitochondrial alternative respiratory pathway and more specifically AOX involved in Cd-induced responses of *A. thaliana* plants exposed to sublethal concentrations?

In addition to AOX, alternative NAD(P)H dehydrogenases (NDs) contribute to the highly branched plant mitochondrial ETC by bypassing complex I and transferring electrons to ubiquinone without pumping protons across the inner membrane (Rasmusson *et al*. 2008). The coordinated expression of genes encoding these enzymes and AOX during abiotic stress conditions (Clifton *et al*. 2005) suggests a coordinated functioning of both components in a condensed ETC uncoupled from ATP synthesis (Vanlerberghe 2013). Further down the ETC, uncoupling proteins (UCPs) dissipate the proton

gradient over the inner mitochondrial membrane, thereby shortcutting the ATP synthase complex and thus affecting ATP yield. Several observations suggest a role for UCPs mediating cellular tolerance to oxidative stress (Nogueira *et al*. 2011), which suggests their involvement in the Cd-induced oxidative challenge as well.

Whereas previous research results support a role for AOX and alternative respiration in mediating responses to highly toxic Cd levels (Keunen *et al*. 2011 and references therein), information on their involvement during an environmentally realistic and sublethal exposure setup is scarce. Therefore, the effects of exposure to 5 or 10 µM Cd on the alternative respiratory chain were studied in wild-type *A. thaliana* roots and leaves using a kinetic exposure setup. Responses to Cd were studied at 2, 24, 48 and 72 h after the exposure started, focussing at transcript (mitochondrial antioxidative enzymes, AOX, ND and UCP) and AOX protein level **(Chapter 4)**.

3. What is the functional significance of ALTERNATIVE OXIDASE1a induction in *A. thaliana* plants during sublethal Cd exposure?

Of the five AOX genes expressed in *A. thaliana*, *AOX1a* is the dominant isoform. The orientating experiment (Chapter 4) supported its potential role in Cd stress responses, as plants were shown to invest in AOX1a at transcript and protein levels. Therefore, Cd-induced responses of wild-type plants were compared to those of plants lacking functional AOX1a (*aox1a* knockout mutants) at different cellular functional levels. First, the functional significance of AOX1a induction in Cd-exposed *A. thaliana* was studied in relation to the oxidative challenge. After 24 and 72 h exposure, responses between both genotypes were compared at molecular and metabolic level in the leaves to reveal the potential involvement of AOX1a in Cd-induced mitochondrial signalling pathways **(Chapter 5)**. Finally, the role of AOX1a in maintaining metabolic homeostasis was investigated in roots and leaves of intact *A. thaliana* plants **(Chapter 6)**. The goal of this final chapter was twofold as it aimed to unravel (1) how sublethal Cd exposure affects primary plant metabolism over time (24 and 72 h) and (2) if and how AOX1a modulates this metabolic response in *A. thaliana*.

REFERENCES

Clemens, S., Aarts, M.G.M., Thomine, S. and Verbruggen, N. (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci*, **18**, 92–99.

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A. and Whelan, J. (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol Biol*, **58**, 193–212.

Cuypers, A., Keunen, E., Bohler, S., Jozefczak, M., Opdenakker, K., Gielen, H., Vercampt, H., Bielen, A., Schellingen, K., Vangronsveld, J. and Remans, T. (2012) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling, in: Gupta, D.K., Sandalio, L.M. (Eds.), Metal toxicity in plants: Perception, signaling and remediation. Springer-Verlag GmbH, Berlin, Heidelberg, pp. 65–90.

Cvetkovska, M. and Vanlerberghe, G.C. (2012) Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol*, **195**, 32–39.

Keunen, E., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2011) Metal-induced oxidative stress and plant mitochondria. *Int J Mol Sci*, **12**, 6894–6918.

Nogueira, F.T.S., Sassaki, F.T. and Maia, I.G. (2011) *Arabidopsis thaliana* Uncoupling Proteins (AtUCPs): insights into gene expression during development and stress response and epigenetic regulation. *J Bioenerg Biomembr*, **43**, 71–79.

Rasmusson, A.G., Geisler, D.A. and Møller, I.M. (2008) The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. *Mitochondrion*, **8**, 47–60.

Smeets, K., Ruytinx, J., Semane, B., Van Belleghem, F., Remans, T., Van Sanden, S., Vangronsveld, J. and Cuypers, A. (2008) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environ Exp Bot*, **63**, 1–8.

Vanlerberghe, G.C. (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci*, **14**, 6805–6847.

CHAPTER 3

Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged?

Els Keunen, Sascha Truyens, Liesbeth Bruckers, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2011) Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged? Plant Physiology and Biochemistry, 49, 1084–1091.

ABSTRACT

Plants exposed to cadmium (Cd) show morphological and physiological disorders. To increase our knowledge regarding Cd-induced signalling, most often the effects of acute exposure are investigated. However, this does not allow in-depth analysis of morphological effects. Therefore, we chronically exposed *Arabidopsis thaliana* plants to environmentally realistic Cd concentrations (5 or 10 μM) and, using a described phenotypic framework methodology, we determined the impact of Cd on the plant's ability to complete its life cycle and produce germinative seeds. Visible Cd-induced morphological changes were observed within a short exposure period, with chlorotic and anthocyanous leaf colouring occurring dose-dependently. Although rosette growth was severely reduced in Cd-exposed plants, all plants were able to emerge inflorescences and produce siliques containing germinative seeds, thus confirming the non-lethality of the used Cd concentrations. Although the growth inhibition of Cd-exposed plants was dependent on the dose, both concentrations had similar effects on inflorescence height and silique counts. In conclusion, vegetative growth of plants chronically exposed to Cd is inhibited in a concentration-dependent manner. However, the effect on plant regeneration is clearly stress-determined but independent on the Cd concentration applied. In *A. thaliana*, vegetative and reproductive growth are differentially influenced by Cd.

Keywords:

Arabidopsis thaliana, cadmium phytotoxicity, development, phenotypic analysis.

3.1. INTRODUCTION

Worldwide, the metal industry and agricultural use of metal-containing fertilisers and pesticides have contributed significantly to metal pollution, posing serious threats to our environment. Contamination with cadmium (Cd), a non-essential element, can lead to decreased crop production and biodiversity. Plants growing on Cd-polluted soils suffer from reduced growth, disturbed photosynthesis and transpiration (Sanità di Toppi and Gabbrielli 1999, Benavides *et al*. 2005, Cuypers *et al*. 2009). Beginning at the plant level, Cd can accumulate in the food chain, thus causing serious concerns for human health (Adriano 2001).

At the cellular level, Cd can cause oxidative stress (Chaoui and El Ferjani 2005, Smeets *et al*. 2005, Garnier *et al*. 2006, Smeets *et al*. 2008a, 2009, Cuypers *et al*. 2011). To investigate the mechanisms involved in cellular and molecular signalling responses to Cd stress, short-term exposure to environmentally realistic Cd concentrations is a recommended experimental setup. Previous studies have shown that *Arabidopsis thaliana* seedlings grown on hydroponics (Smeets *et al*. 2008b) can efficiently cope with 24 h exposure to 5 or 10 µM Cd, as suggested by the coordinated oxidative stress-related defence responses observed (Smeets *et al*. 2008a). This indicates that before any morphological effects become visible, toxic internal Cd concentrations are interfering at molecular and biochemical levels (Cuypers *et al*. 2011, Lagriffoul *et al*. 1998). However, to study cellular and molecular effects in a larger context including macroscopic effects, prolonged exposure to Cd is inevitable.

As described by Boyes *et al*. (2001), altered environmental stress conditions may affect a number of traits, finally resulting in morphological changes and/or altered developmental progression. Morphological effects resulting from Cd exposure may not be visible within the short time periods often used in studies focusing on cellular and molecular responses (Suzuki 2005, Sarry *et al*. 2006, Smeets *et al*. 2008, 2009, Remans *et al*. 2010, Cuypers *et al*. 2011). However, chlorosis and anthocyanous colouring of the leaves are two well-known effects frequently observed with prolonged exposure to moderate and high Cd concentrations (Sanità di Toppi and Gabbrielli 1999, Van Belleghem *et al*. 2007). Also, Cd exposure was suggested to induce or accelerate leaf senescence (Srivastava and Jaiswal 1989, Sandalio *et al*. 2001).

Boyes *et al*. (2001) have described a method for phenotyping plants based on a series of well-defined stages in *A. thaliana* growth and development. The growth stages (Boyes *et al*. 2001) span the entire life cycle of the plant, which maximises the potential to detect subtle changes affecting only limited developmental aspects. Within this framework methodology, our experimental aim was to identify and interpret phenotypic effects in *A. thaliana* seedlings continuously exposed to environmentally realistic Cd concentrations (Krznaric *et al*. 2009), thereby ensuring the non-lethality of the Cd concentrations used to study moderate toxicity within a shorter exposure period (Smeets *et al*. 2009). The Cd concentrations applied were based on those measured in the pore water of sandy soils in specific polluted regions in Belgium (Krznaric *et al*. 2009). Earlier results have shown that prolonged exposure to similar concentrations resulted in growth reduction and chlorosis (Semane *et al.* 2007, Van Belleghem *et al*. 2007). However, the effects at the regenerative phase were not investigated. Therefore, we conducted a long-term experiment exposing 17-days-old *Arabidopsis* plants chronically to 0, 5 or 10 µM Cd in a hydroponical system. Phenotypic characteristics were observed and evaluated by comparing Cd-exposed and unexposed plants until seeds were produced. The ultimate aim of this experiment was dual: to confirm the non-lethal outcome of chronic 5 and 10 µM Cd exposure in *A. thaliana* and to study the effect of both concentrations on vegetative biomass production and regenerative growth. Based on previous results (Semane *et al.* 2007, Van Belleghem *et al*. 2007), we hypothesise that plants exposed to 5 or 10 µM Cd do not only survive the imposed stress factor, but are even able to complete their life cycle. Since measurements were taken from the same plants at different time points, statistical modelling approaches for repeated data were applied to verify the significance of the observed effects on vegetative and reproductive growth of Cd-exposed *Arabidopsis* seedlings.

3.2. MATERIALS AND METHODS

3.2.1. Plant material and cadmium treatment

Wildtype *A. thaliana* seedlings (Columbia ecotype) were grown on hydroponics as described by Smeets *et al*. (2008b), except that purified sand was used instead of rock wool (Remans *et al*. 2010). A modified Hoagland nutrient solution was used and contained macronutrients [505 µM KNO_3, 150 µM $Ca(NO_3)_2.4H_2O$, 100 µM $NH_4H_2PO_4$ and 100 µM $MgSO_4.7H_2O$], micronutrients [4.63 µM H_3BO_3, 0.91 µM $MnCl_2.4H_2O$, 0.03 µM $CuSO_4.5H_2O$, 0.06 µM $H_2MoO_4.H_2O$ and 0.08 µM $ZnSO_4.7H_2O$], 1.64 µM $FeSO_4.7H_2O$ and 0.81 µM Na_2-EDTA. Growth conditions were set at a 12 h photoperiod, 65% relative humidity and day/night temperatures of 22 °C and 18 °C respectively. Light was provided by Philips GreenPower LED modules. A combination of blue, red and far-red modules was used to obtain a spectrum simulating the photosynthetic active radiation (PAR) of sunlight. The PAR provided at the rosette level was 170 µmol m^{-2} s^{-1}. At the age of 17 days, plants were exposed to $CdSO_4$ (0, 5 or 10 µM) supplied to the roots. Throughout the entire time course of the experiment (day 17 to day 64), the plants were continuously exposed to Cd or grown under control conditions. New Hoagland solution with or without Cd was provided once every week to ensure and maintain nutrient availability (Semane *et al*. 2007, Dauthieu *et al*. 2009).

3.2.2. Hydroponics-based phenotypic analysis

The measurements to assess the growth and development of both unexposed and Cd-exposed plants were started the day before exposing the plants to $CdSO_4$. Rosette growth and leaf development were monitored daily and as soon as inflorescence emerged, the height of the inflorescence and number of siliques per plant was also followed until a clear distinction between unexposed and Cd-exposed plants was observed. In Table 3.1, an overview of the used growth stages adapted from the soil-based phenotypic analysis platform described by Boyes *et al*. (2001) is shown. To achieve our goal, we studied the influence of Cd exposure on principal growth stages 1, 3 and 5. Principal growth stage 1 defines leaf development, growth stage 3 is related to rosette growth and growth stage 5 describes the emergence of the inflorescence. Thus, vegetative

plant growth is studied here via comparative analysis of growth stages 1 and 3 in Cd-exposed and unexposed plants, while growth stage 5 is related to regeneration.

Table 3.1. *Arabidopsis thaliana growth stages for the used hydroponics-based phenotypic analysis platform. The growth stages used in this analysis were adapted from the soil-based phenotypic analysis platform described by Boyes et al. (2001). Both vegetative (leaf development and rosette growth) and regenerative (inflorescence emergence) growth characteristics of Arabidopsis thaliana were studied. The leaf development analysis started with stage 1.06 since plants had six rosette leaves larger than 1 mm at the beginning of Cd exposure. Also, the regenerative aspect was expanded with the determination of inflorescence height and silique counts.*

Stage	Description
Principal growth stage 1	**Leaf development**
1.06	6 rosette leaves > 1 mm in length
1.08	8 rosette leaves > 1 mm in length
1.10	10 rosette leaves > 1 mm in length
1.12	12 rosette leaves > 1 mm in length
1.14	14 rosette leaves > 1 mm in length
1.16	16 rosette leaves > 1 mm in length
1.18	18 rosette leaves > 1 mm in length
1.20	20 rosette leaves > 1 mm in length
1.22	22 rosette leaves > 1 mm in length
1.24	24 rosette leaves > 1 mm in length
1.26	26 rosette leaves > 1 mm in length
1.28	28 rosette leaves > 1 mm in length
Principal growth stage 3	**Rosette growth**
3.50	Rosette measures 50% of its final size
3.70	Rosette measures 70% of its final size
3.90	Rosette growth is finished
Principal growth stage 5	**Emergence of inflorescence**
5.10	Emergence of the inflorescence meristem

3.2.3. Statistical analysis via a modelling approach

Since measurements were taken from the same plants at different time points (*i.e.* repeated measures), a correction for the correlation between multiple

observations is necessary. Mixed-effects models (Davidian and Giltinan 1995) and generalised estimating equations (Liang and Zeger 1986) are flexible and powerful tools for the statistical analysis of repeated measures. Lindstrom and Bates (1990) and Pinheiro and Bates (1995) propose the following logistic nonlinear mixed model for repeated Gaussian data, relating a response 'y' to a covariate (the age of the plant) via a sigmoidal or S-shaped function:

$$Y_{ij} = \frac{\beta_1 + u_{i1}}{1 + \exp\left[-\left(\frac{t_{ij} - \beta_2}{\beta_3}\right)\right]} + \varepsilon_{ij}$$

In this equation, Y_{ij} represents the j^{th} measurement on the i^{th} plant, t_{ij} is the corresponding age (in days), β_1, β_2 and β_3 are the fixed-effects parameters, u_{i1} represent the random-effect parameters assumed to be independent and identically distributed as $N(0, \sigma^2_u)$ and ε_{ij} are the residual errors assumed to be independent and identically distributed as $N(0, \sigma^2_e)$ and independent of the u_{i1}. The random effect in this model introduces correlation between repeated measures taken on the same plant. The random-effect parameters u_{i1} enter the model linearly. As a result, the marginal mean simplifies to:

$$E(Y_{ij}) = \frac{\beta_1}{1 + \exp\left[-\left(\frac{t_{ij} - \beta_2}{\beta_3}\right)\right]}$$

This nonlinear mixed model provides a framework to describe the underlying biological processes giving rise to our data since the fixed parameters have a clear interpretable meaning. Parameter β_3 is a shape index. If $\beta_3 > 0$, parameter β_1 represents the horizontal asymptote as $t \rightarrow \infty$, while 0 is the horizontal asymptote as $t \rightarrow -\infty$. Parameter β_2 is the inflection point; at this age the response equals $\beta_1/2$. The model specified above was used to fit leaf development (Figure 3.2B), rosette diameter (Figure 3.3B), inflorescence height (Figure 3.5B) and number of siliques (Figure 3.6B). As parameter β_1 is indicative for the maximum of the fitted curves, the difference in this parameter between unexposed and 5 or 10 µM Cd-exposed plants is compared for data interpretation (Table 3.3). All models were fitted with PROC NLMIXED in SAS, version 9.2 (SAS Institute Inc., Cary, NC, USA).

The fit of the model to the observed data was compared with the fit of models proposed by other authors (Peek *et al*. 2002, Guan *et al*. 2008) and with the fit of a quadratic or cubic trend with age. According to Akaike and Schwarz

information criteria (Burnham and Anderson 2004), the model used in our analyses is for all responses the best fitting model.

For each response, we determined the age when differences between unexposed and Cd-exposed plants become statistically significant via pair wise comparisons (*i.e.* 0 versus 5 µM Cd, 0 versus 10 µM Cd and 5 versus 10 µM Cd) at different ages. These comparisons were adjusted for multiple testing by a Bonferroni correction in a way that the family wise error rate at age t is at most 5%.

The emergence of the inflorescence meristem (Figure 3.4B) is a binary (0 or 1) response for which generalised estimating equations (GEE) were used to model the correlated binary sequences. This approach requires a correct specification of the marginal distribution and working assumptions about the association structure. The following model with an autoregressive working correlation structure was used:

$$E(Y_{ij}) = \frac{\exp[\beta_0 + \beta_1 t_{ij}]}{1 + \exp[\beta_0 + \beta_1 t_{ij}]}$$

The interpretation of the parameter β_1 in this model is in terms of the odds ratio. The odds for inflorescence meristems is defined as the ratio of two probabilities: $p(Y_{ij}=1)/p(Y_{ij}=0)$. The logistic regression model describes the log odds as a linear function of age, *i.e.* $\log(p(Y_{ij}=1)/p(Y_{ij}=0)) = \beta_0 + \beta_1 t_{ij}$. For $\beta_1 > 0$, the odds increases every day with a factor $\exp[\beta_1]$. Thus, the odds ratio (the ratio of the odds at day t_{ij} and the odds at day t_{ij+1}) is equal to $\exp[\beta_1]$. The larger parameter β_1, the faster the (log) odds increases with age; and the steeper the curve of $p(Y_{ij}=1)$ as a function of age. The model specified above was fitted for inflorescence meristems (Figure 3.4B) with PROC GENMOD in SAS, version 9.2 (SAS Institute Inc., Cary, NC, USA).

3.3. RESULTS

3.3.1. Stress-induced vegetative changes in Cd-exposed Arabidopsis thaliana

In the current study, signs of leaf chlorosis were already visible after four days of exposure to both Cd concentrations. In plants exposed to the lowest Cd concentration, leaf chlorosis worsened throughout the experiment, but leaf density remained high (Figure 3.1). After 21 days of exposure, anthocyanous-coloured leaves were apparent on plants exposed to 10 µM Cd, which also had a lower leaf density as shown in Figure 3.1B. Root growth was inhibited in plants exposed to both Cd concentrations as compared to the control, with reductions of ~ 45% and ~ 65% in 5 and 10 µM Cd-exposed plants respectively at the moment of finished rosette growth (data not shown).

Figure 3.1. *Representative pictures of the appearance and height of Arabidopsis thaliana plants continuously exposed to 5 or 10 µM $CdSO_4$ or grown under control conditions. (A) Rosette appearance. For each group, pictures were taken at day 28. (B) Inflorescence height and leaf density. For each group, pictures were taken at day 56.*

We studied leaf development and rosette growth as morphological traits of vegetative growth possibly influenced by Cd exposure. Leaf development is described by principal growth stage 1 (Boyes *et al*. 2001), where the last two digits indicate the number of rosette leaves longer than 1 mm (Table 3.1). In the current study, this growth stage was described by a broad range from 1.06 at the beginning of Cd exposure (day 17) to 1.28 in plants exposed to 10 µM Cd (Table 3.2). As shown in Figure 3.2A, leaf development was affected by Cd in a dose-dependent way. Starting from the age of 21 days (*i.e.* four days of Cd exposure), the difference between unexposed and 10 µM Cd-exposed plants was significant ($p < 0.05$), while the difference between 0 and 5 µM Cd-exposed seedlings only became significant at age 32 days ($p < 0.05$) (Figure 3.2A).

Next to leaf development, we also investigated rosette growth described by principal growth stage 3 (Boyes *et al*. 2001) (Tables 3.1 and 3.2). As shown in Figure 3.3A, the average rosette diameter of plants exposed to 5 µM Cd decreased with approximately 40%, while this reduction was more pronounced after chronic exposure to 10 µM Cd (about 60% at finished rosette growth). Remarkable is that differences already became obvious after a short exposure time for the highest Cd concentration (inset in Figure 3.3A), with significant differences between 0 and 10 µM Cd starting at the age of 19 days (*i.e.* two days of Cd exposure, $p < 0.05$). For the lower Cd concentration, the difference with unexposed plants was statistically significant starting from day 21 (Figure 3.3), indicating that Cd exposure negatively affects rosette growth and that this effect is dose-dependent. Interestingly, while rosette growth of 10 µM Cd-exposed plants was finished at approximately 33 days (growth stage 3.90, Table 3.2), leaf development continued for another three days (growth stage 1.28, Table 3.2). This was not the case for plants exposed to 5 µM Cd and plants grown under control conditions, where all leaves were formed before rosette growth finished (Table 3.2).

Table 3.2. *Arabidopsis thaliana growth stages for the hydroponics-based phenotypic analysis of Cd-exposed plants compared to plants grown under control conditions. The day a specific growth stage was reached by 30 biological replicates in each group was monitored from the day before Cd exposure (day 16) until clear differences in reproductive growth (day 64) were observed between unexposed and Cd-exposed plants. For each principal growth stage until inflorescence as described by Boyes et al. (2001), data are given as the average day from date after sowing ± S.E. Growth stage 1 defines leaf development, growth stage 3 is related to rosette growth and growth stage 5 describes the emergence of inflorescence.*

[$CdSO_4$]	**0 µM**	**5 µM**	**10 µM**
Principal growth stage 1		Mean ± S.E.	
1.06	16.00 ± 0.00	16.00 ± 0.00	16.00 ± 0.00
1.08	18.10 ± 0.06	18.17 ± 0.07	18.40 ± 0.10
1.10	19.57 ± 0.09	19.93 ± 0.13	20.50 ± 0.12
1.12	21.33 ± 0.09	21.83 ± 0.14	22.47 ± 0.10
1.14	22.90 ± 0.11	23.17 ± 0.14	23.67 ± 0.15
1.16	24.30 ± 0.12	24.60 ± 0.17	24.97 ± 0.17
1.18	25.53 ± 0.09	25.90 ± 0.22	26.73 ± 0.19
1.20	26.63 ± 0.14	27.23 ± 0.29	28.63 ± 0.33
1.22	28.13 ± 0.22	28.70 ± 0.46	30.45 ± 0.36
1.24	29.68 ± 0.25	30.57 ± 0.48	33.07 ± 0.36
1.26	31.07 ± 0.23	32.30 ± 0.46	35.04 ± 0.36
1.28	32.14 ± 0.20	33.31 ± 0.45	36.88 ± 0.27
Principal growth stage 3		Mean ± S.E.	
3.50	24.64 ± 0.20	22.03 ± 0.33	19.08 ± 0.48
3.70	28.68 ± 0.25	26.23 ± 0.54	22.97 ± 0.41
3.90	38.39 ± 0.40	37.33 ± 0.66	33.37 ± 1.13
Principal growth stage 5		Mean ± S.E.	
5.10	39.21 ± 0.35	36.17 ± 0.54	38.47 ± 0.63

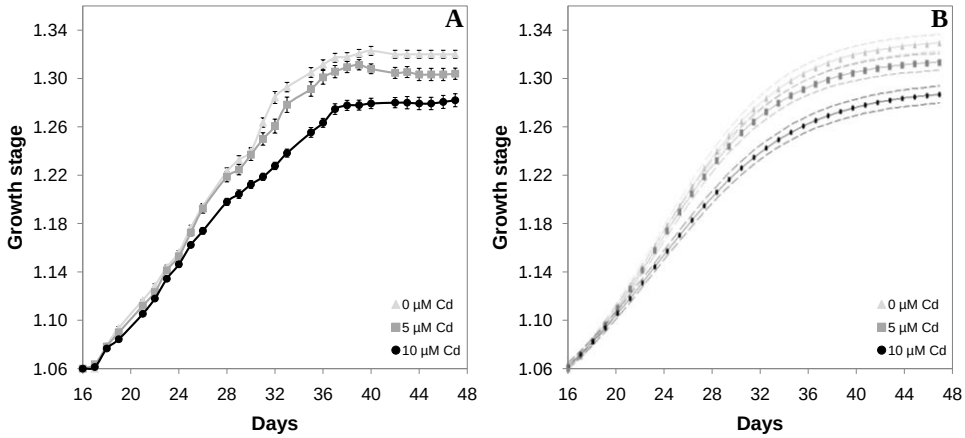


Figure 3.2. *Exposure to Cd has an influence on the leaf development of Arabidopsis thaliana plants continuously exposed to 5 or 10 µM $CdSO_4$ or grown under control conditions. (A) The average growth stage related to the number of rosette leaves (1.xx with xx indicating the number) over time is shown; data are given as the average per day after sowing ± S.E. for 30 biological replicates per group. (B) The average fitted profiles based on a nonlinear mixed model with confidence intervals for unexposed and Cd-exposed plants.*

In Figures 3.2B and 3.3B, the average fitted profiles with confidence intervals based on a nonlinear mixed model are shown for leaf development and rosette diameter respectively. It is clear that the confidence intervals of the curves fitted for 0, 5 and 10 µM Cd-exposed seedlings do not overlap (except for 0 and 5 µM Cd-induced effects on leaf development). Parameter β_1 of the model represents the maximum of the fitted curves (Table 3.3). For both leaf development and rosette diameter, the difference in β_1 was significant for unexposed versus Cd-exposed plants and also for 5 versus 10 µM Cd (Table 3.3). This underlines that the impact of Cd exposure on vegetative growth studied by leaf development and rosette diameter is dependent on the concentration applied.

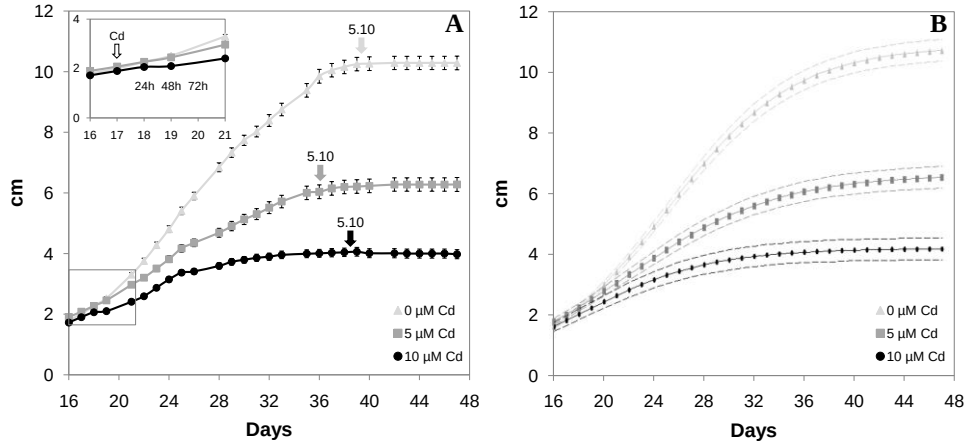


Figure 3.3. *Exposure to Cd has an influence on the rosette diameter of Arabidopsis thaliana plants continuously exposed to 5 or 10 µM $CdSO_4$ or grown under control conditions. (A) The average rosette diameter (cm) over time is shown; data are given as the average per day after sowing ± SE for 30 biological replicates per group. Exposure to Cd started at day 17. Arrows indicate the average time at which growth stage 5.10 (first inflorescence meristems visible) was reached. The inset shows the short-term effects of Cd exposure in the time period regularly studied in kinetic experiments, ranging from 24 to 72 h. (B) The average fitted profiles based on a nonlinear mixed model with confidence intervals for unexposed and Cd-exposed plants.*

3.3.2. Impact of Cd exposure on inflorescence emergence in Arabidopsis thaliana

The altered vegetative growth under Cd stress conditions (Figures 3.2 and 3.3) could have an impact on the emergence of the inflorescence meristem. Therefore, the average time at which plants exposed to 5 or 10 µM Cd and unexposed plants had visible inflorescence meristems and thus reached growth stage 5.10 (Boyes *et al*. 2001) was determined (Table 3.2) and indicated in Figure 3.3A. For the control, stage 5.10 occurred nearly immediately after rosette diameter growth finished (Figure 3.3A). While this was also true for 5 µM Cd-exposed plants, they needed less time to reach stage 5.10 as compared to the control (Table 3.2). Remarkably, plants exposed to the highest Cd concentration only reached 5.10 approximately five days after the rosette diameter reached its maximum (Figure 3.3A, Table 3.2).

Table 3.3. *Overview of the statistical interpretation of the modelling approaches used in this study. A nonlinear mixed model was used to fit leaf development, rosette diameter, inflorescence height and silique counts. For each response, parameter β_1 of the model indicates the maximum of the fitted curves. The p-values represent the significance of the difference in β_1 of both unexposed and 5 or 10 µM Cd-exposed Arabidopsis thaliana seedlings. For the emergence of the inflorescence meristem, a generalised estimating equations model was fitted where parameter β_1 represents the slope of the fitted curves.*

[$CdSO_4$]	**0 versus 5 µM**	**0 versus 10 µM**	**5 versus 10 µM**
	p-value for the difference in β_1 **~ maximum**		
VEGETATIVE GROWTH			
Leaf development	0.0024	< 0.0001	< 0.0001
Rosette diameter	< 0.0001	< 0.0001	< 0.0001
	p-value for the difference in β_1 **~ maximum**		
REGENERATIVE GROWTH			
Inflorescence height	< 0.0001	< 0.0001	0.2610
Silique counts	< 0.0001	0.0001	0.8759
	p-value for the difference in β_1 **~ slope**		
Inflorescence meristem	0.0004	0.0012	0.8165

The percentage of plants possessing visible inflorescence meristems started to increase earlier in Cd-exposed plants as compared to the control (Figure 3.4A). Fitting the curves based on a generalised estimating equations model revealed a similar increase for both Cd concentrations, while the increment in control plants was steeper (Figure 3.4B). This is underlined by the similar β_1 values for 5 and 10 µM Cd-exposed seedlings (Figure 3.4B), which clearly differed from the control group (Table 3.3).

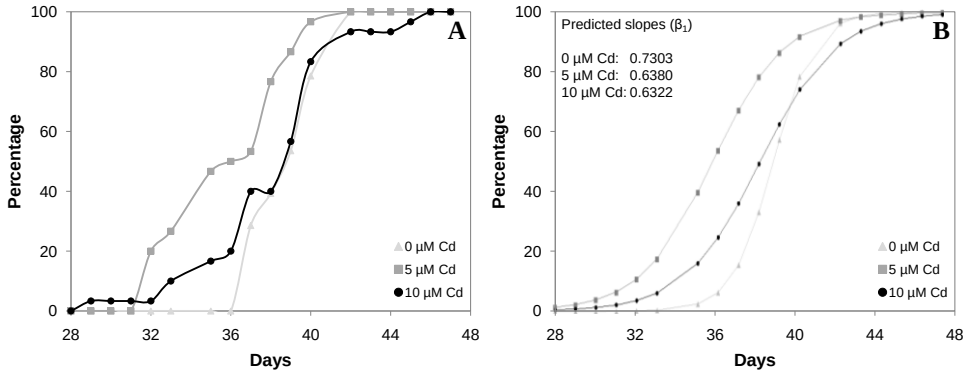


Figure 3.4. *Exposure to Cd has an influence on inflorescence emergence in Arabidopsis thaliana plants continuously exposed to 5 or 10 µM $CdSO_4$ or grown under control conditions. (A) The percentage of plants with inflorescence meristems over time. (B) The average fitted profiles based on a generalised estimating equations model for unexposed and Cd-exposed plants.*

3.3.3. Are Cd-exposed Arabidopsis thaliana plants reproductively challenged?

A first evidence for the sublethal character of the Cd concentrations applied was revealed by the observation that all plants in all three conditions were able to form inflorescence meristems (Figure 3.4), indicative of the start of reproductive growth. Starting from the age of 44 days, *i.e.* 27 days of Cd exposure, the inflorescence of Cd-exposed plants was significantly shorter as compared to plants grown under control conditions (Figure 3.5, $p < 0.05$). The ability of Cd-exposed plants to complete a life cycle from seed to new germinative seeds was confirmed by the formation of seed-containing siliques (Figure 3.6). Exposure to Cd however dramatically decreased the number of siliques with ~ 90% as compared to the control, where at 64 days, the silique count was still increasing (Figure 3.6A). The differences in silique counts between Cd-exposed and unexposed plants were significant starting from age 49 (for 0 versus 5 µM Cd) and 50 (for 0 versus 10 µM Cd) days (Figure 3.6, $p < 0.05$). While the number of seeds produced was not assessed in this study, seed germinative capacity was tested and shown to be equal for both seeds from Cd-exposed and unexposed plants (data not shown).

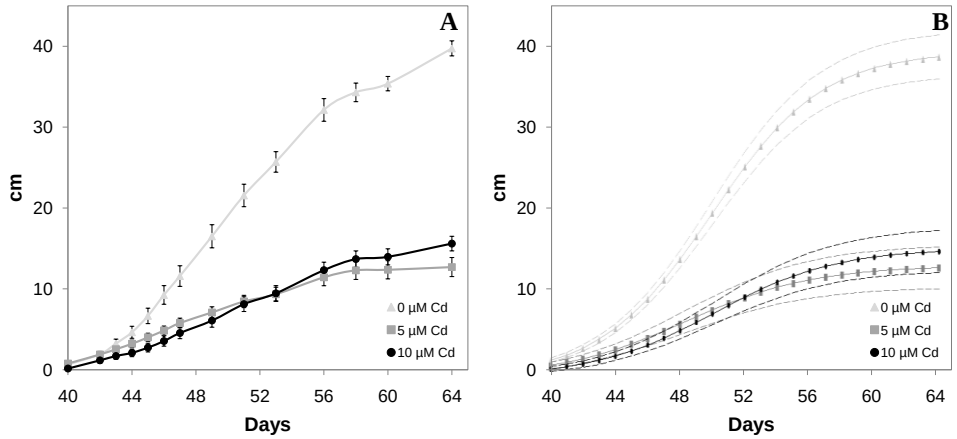


Figure 3.5. *Exposure to Cd has an influence on the inflorescence height of Arabidopsis thaliana plants continuously exposed to 5 or 10 µM $CdSO_4$ or grown under control conditions. (A) The average inflorescence height (cm) over time is shown; data are given as the average per day after sowing ± S.E. for 30 biological replicates per group. (B) The average fitted profiles based on a nonlinear mixed model with confidence intervals for unexposed and Cd-exposed plants.*

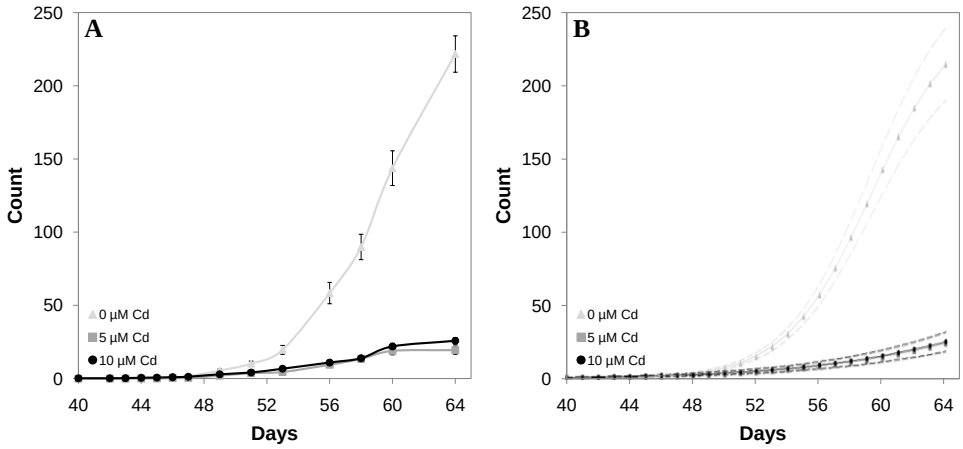


Figure 3.6. *Exposure to Cd has an influence on the number of siliques produced by Arabidopsis thaliana plants continuously exposed to 5 or 10 µM CdSO4 or grown under control conditions. (A) The average silique counts over time are shown; data are given as the average per day after sowing ± S.E. for 30 biological replicates per group. (B) The average fitted profiles based on a nonlinear mixed model with confidence intervals for unexposed and Cd-exposed plants.*

In Figures 3.5B and 3.6B, the average fitted curves with confidence intervals based on a nonlinear mixed model are shown for inflorescence height and silique count respectively, with parameter β_1 again representing the maximum (Table 3.3). It is clear that the confidence intervals of the curves fitted for 5 and 10 µM Cd-exposed seedlings overlap (Figures 3.5B and 3.6B). Thus, the impact of Cd exposure on regenerative growth is similar for both concentrations applied as indicated by the p-values, which is also the case for the inflorescence meristem (Table 3.3).

From our observations, it is obvious that Cd affects vegetative growth measured as leaf development and rosette growth in a dose-dependent manner. However, the formation of seed-containing siliques defined as the endpoint of regenerative growth, is equally diminished in both 5 and 10 µM Cd-exposed plants. Exposure to Cd thus has a different influence on vegetative and regenerative growth of *Arabidopsis* plants.

3.4. DISCUSSION

A better understanding of metal-induced effects at different cellular functional levels is useful for developing strategies to grow non-food crops on polluted agricultural soils (Cuypers *et al*. 2011). In the past, different research setups were used to study Cd toxicity in plants, where the choice of Cd concentrations and time of exposure often depended on the desired outcome. When studying the effects of Cd on plant growth and physiology, the concentrations used can vary over the micromolar to millimolar range (Reddy and Prasad 1995, Lagriffoul *et al*. 1998, Ci *et al*. 2009) to very high concentrations that are even misleading in the field (for a review see Sanità di Toppi and Gabbrielli 1999). During recent years, research has shifted from focusing on the impact of Cd exposure on morphology and/or physiology at the whole plant level to cellular and even molecular responses mediated by signalling processes, thereby implying the need for acute Cd exposure. Within this short exposure period, neither growth reduction nor visible toxicity symptoms were observed when exposing three-week-old *Arabidopsis* seedlings to 5 or 10 µM Cd during 24 h (Cuypers *et al*. 2011).

To incorporate cellular and molecular changes into a larger macroscopic context, we investigated the morphological effects of long-term exposure to environmentally realistic Cd concentrations using a phenotypic analysis framework described by Boyes *et al*. (2001). This framework methodology is adapted from the BBCH scale (Lancashire *et al*. 1991) for use in the phenotypic analysis of *Arabidopsis*. It is composed of 30 growth stages covering complete plant development from seed imbibition to flowering and seed maturation. In this study, plants were exposed starting from the age of 17 days. Therefore, phenotypic analysis was only carried out starting the day before the start of the Cd exposure, thereby overcoming the need for comparative seed development analysis. When comparing numerical data obtained in our analyses to those of Boyes *et al*. (2001), slight differences were noticed. At the start of Cd exposure, all plants in this study reached growth stage 1.06, indicating the presence of six rosette leaves (Tables 3.1 and 3.2). The plants showed more vegetative growth as compared by Boyes *et al*. (2001), which could be explained by different growth conditions.

Both root and leaf growth (Figures 3.1 and 3.2) were reduced by exposing *A. thaliana* to 5 or 10 µM Cd as compared to the control. At the age of 28 days (Figure 3.1A), the rosette diameter of plants grown under control conditions is still increasing, while plants exposed to the highest Cd concentration are already in the final stage (Figure 3.3A). As shown by Dauthieu *et al*. (2009), Cd uptake and distribution in *A. thaliana* chronically exposed to low concentrations in the nanomolar range depends on plant growth. In our study, plants exposed to 5 or 10 µM Cd could reduce root and/or leaf growth to limit Cd uptake. However, all Cd-exposed plants were able to complete their life cycle and thus produce siliques containing germinative seeds (Figure 3.6A). The strong reduction in silique counts of 5 or 10 µM Cd-exposed plants (Figure 3.6A) is indicative for the stress the plants are experiencing.

For leaf development, rosette diameter and inflorescence height, the observed data shown in Figures 3.2A, 3.3A and 3.5A correlated well with the predicted values for β_1 by the model (Figures 3.2B, 3.3B and 3.5B). However, for siliques (Figure 3.6), the predicted β_1 values were higher as compared to the observed data. This could be explained by the fact that the plateau phase in silique counts was not reached during the time course of the experiment. However, it was clear

that Cd exposure affected the number of siliques independent of the dose confirmed by similar β_1 values for 5 and 10 µM Cd-exposed seedlings (data not shown).

Although both Cd concentrations affected the formation of inflorescence meristems (Figure 3.4), inflorescence height (Figure 3.5) and silique counts (Figure 3.6) in a similar way, the effects on leaf development (Figure 3.2) and rosette growth were dose-dependent (Figure 3.3). This suggests that Cd exposure affects vegetative and reproductive growth of *A. thaliana* in a different way, which is in line with the results of Ismail (2008). Exposing bean plants to 100 or 200 µM $CdCl_2$ had dose-dependent effects on the number of leaves per plant. However, the inflorescence height decreased with the same order of magnitude after exposure to both Cd concentrations (Ismail 2008). Sandalio *et al*. (2001) studied Cd-induced changes in the growth of pea plants and observed greater rosette area reductions with increasing exposure concentrations. This hypothesis is also confirmed by two end-point measurements performed in the current study, where the plant inflorescence height to the first silique and the number of branches on the main bolt both decreased with ~ 50% in both 5 and 10 µM Cd-exposed plants as compared to the control (data not shown).

The earlier emergence of inflorescence meristems indicated in Figure 3.4A and the fact that 5 µM Cd-exposed plants needed less time to reach growth stage 5.10 (Table 3.2) are in line with the suggested Cd-induced accelerated senescence in plants (Srivastava and Jaiswal 1989, Sandalio *et al*. 2001). However, the transition from vegetative to reproductive growth was unaffected by exposure to 10 µM Cd in accordance with the results of Maistri *et al*. (2011). They have shown that Cd affects the expression of *ELF4*, a circadian clock gene involved in the genetic control of flowering in *Arabidopsis*, which could influence the development of plants chronically exposed to Cd in the field (Maistri *et al*. 2011).

Even within a short exposure period ranging from 24 to 72 h (see inset in Figure 3.3A), differences between both Cd concentrations were apparent. Plants exposed to 10 µM Cd showed morphological disturbances after only 72 h, whereas no differences were observed after 24 h of metal exposure, which is in accordance with previous results (Cuypers *et al*. 2011). These results support our experimental setup to investigate Cd-induced effects at cellular and

molecular levels by exposing *Arabidopsis* seedlings to clearly toxic but sublethal Cd concentrations.

In conclusion, we confirmed the sublethal character of the Cd concentrations measured in the pore water of polluted sandy soils in Belgium (Krznaric *et al*. 2009). Continuously exposing *A. thaliana* to these metal concentrations reduces their reproductive capacity, but the plants are still able to grow, survive and produce siliques containing germinative seeds. The impact of Cd exposure on the vegetative stadium and thus biomass production is clearly depending on the dose. However, the regenerative growth of *A. thaliana* exposed to environmentally realistic Cd concentrations is negatively influenced independent of the Cd concentration applied and thus stress-determined.

REFERENCES

Adriano, D.C. (2001) Cadmium, in: Adriano, D.C. (Ed.), Trace elements in terrestrial environments. Biogeochemistry, bioavailability, and risks of metals. Springer-Verlag, New York, Berlin, Heidelberg, pp. 263–314.

Benavides, M.P., Gallego, S.M. and Tomaro, M.L. (2005) Cadmium toxicity in plants. *Braz J Plant Physiol*, **17**, 21–34.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R. and Görlach, J. (2001) Growth stage-based phenotypic analysis of Arabidopsis: A model for high throughput functional genomics in plants. *Plant Cell*, **13**, 1499–1510.

Burnham, K.P. and Anderson, D.R. (2004) Multimodel inference: Understanding AIC and BIC in model selection. *Sociol Method Res*, **33**, 261–304.

Chaoui, A. and El Ferjani, E. (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. *C R Biol*, **328**, 23–31.

Ci, D., Jiang, D., Dai, T., Jing, Q. and Cao, W. (2009) Effects of cadmium on plant growth and physiological traits in contrast wheat recombinant inbred lines differing in cadmium tolerance. *Chemosphere*, **77**, 1620–1625.

Cuypers, A., Smeets, K. and Vangronsveld, J. (2009) Heavy metal stress in plants, in: Hirt, H. (Ed.), Plant stress biology. From genomics to systems biology. Wiley-VCH Verlagsgesellschaft GmbH & Co. KGaA., Weinheim, pp. 161–178.

Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J. and Vangronsveld, J. (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J Plant Physiol*, **168**, 309–316.

Dauthieu, M., Denaix, L., Nguyen, C., Panfili, F., Perrot, F. and Potin-Gautier, M. (2009) Cadmium uptake and distribution in *Arabidopsis thaliana* exposed to low chronic concentrations depends on plant growth. *Plant Soil*, **322**, 239–249.

Davidian, M. and Giltinan, D.M. (1995) Nonlinear Models for Repeated Measurement Data, first ed., Chapman & Hall, New York.

Garnier, L., Simon-Plas, F., Thuleau, P., Agnel, J.P., Blein, J.P., Ranjeva, R. and Montillet, J.L. (2006) Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant Cell Environ*, **29**, 1956–1969.

Guan, B.T., Lin, S.T., Lin, Y.H. and Wu, Y.S. (2008) Growth efficiency-survivorship relationship and effects of spacing on relative diameter growth rate of Japanese cedars. *Forest Ecol Manag*, **255**, 1713–1723.

Ismail, M.A. (2008) Involvement of Ca^{2+} in alleviation of Cd^{2+} toxicity in common bean (*Phaseolas vulgaris* L.) plants. *Res J Agr Biol Sci*, **4**, 203–209.

Krznaric, E., Verbruggen, N., Wevers, J.H.L., Carleer, R., Vangronsveld, J. and Colpaert, J.V. (2009) Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd. *Environ Pollut*, **157**, 1581–1588.

Lagriffoul, A., Mocquot, B., Mench, M. and Vangronsveld, J. (1998) Cadmium toxicity effects on growth, mineral and chlorophyll contents, and activities of stress related enzymes in young maize plants (*Zea mays* L.). *Plant Soil*, **200**, 241–250.

Lancashire, P.D., Bleiholder, H., van den Boom, T., Langelüddeke, P., Stauss, R., Weber, E. and Witzenberger, A. (1991) A uniform decimal code for growth stages of crops and weeds. *Ann Appl Biol*, **119**, 561–601.

Liang, K.Y. and Zeger, S.L. (1986) Longitudinal data analysis using generalized linear models. *Biometrika*, **73**, 13–22.

Lindstrom, M.J. and Bates, D.M. (1990) Nonlinear mixed effects models for repeated measures data. *Biometrics*, **46**, 673–687.

Maistri, S., DalCorso, G., Vicentini, V. and Furini, A. (2011) Cadmium affects the expression of *EFL4*, a circadian clock gene in *Arabidopsis*. *Environ Exp Bot*, **72**, 115–122.

Peek, M.S., Russek-Cohen, E., Wait, D.A. and Forseth, I.N. (2002) Physiological response curve analysis using nonlinear mixed models. *Oecologia*, **132**, 175–180.

Pinheiro, J.C. and Bates, D.M. (1995) Approximations to the log-likelihood function in the nonlinear mixed-effects model. *J Comput Graph Stat*, **4**, 12–35.

Reddy, G.N. and Prasad, M.N.V. (1995) Cadmium-induced protein phosphorylation changes in rice (*Oryza sativa* L.) seedlings. *J Plant Physiol*, **145**, 67–70.

Remans, T., Opdenakker, K., Smeets, K., Mathijsen, D., Vangronsveld, J. and Cuypers, A. (2010) Metal-specific and NADPH oxidase dependent changes in lipoxygenase and NADPH oxidase gene expression in *Arabidopsis thaliana* exposed to cadmium or excess copper. *Funct Plant Biol*, **37**, 532–544.

Sandalio, L.M., Dalurzo, H.C., Gómez, M., Romero-Puertas, M.C. and del Río, L.A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J Exp Bot*, **52**, 2115–2126.

Sanità di Toppi, L. and Gabbrielli R. (1999) Response to cadmium in higher plants. *Environ Exp Bot*, **41**, 105–130.

Sarry, J.E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain, A., Bastien, O., Fievet, J.B., Vailhen, D., Amekraz, B., Moulin, C., Ezan, E., Garin, J. and Bourguignon, J. (2006) The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics*, **6**, 2180–2198.

Semane, B., Cuypers, A., Smeets, K., Van Belleghem, F., Horemans, N., Schat, H. and Vangronsveld, J. (2007) Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. *Physiol Plant*, **129**, 519–528.

Smeets, K., Cuypers, A., Lambrechts, A., Semane, B., Hoet, P., Van Laere, A. and Vangronsveld, J. (2005) Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. *Plant Physiol Biochem*, **43**, 437–444.

Smeets, K., Ruytinx, J., Semane, B., Van Belleghem, F., Remans, T., Van Sanden, S., Vangronsveld, J. and Cuypers, A. (2008a) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environ Exp Bot*, **63**, 1–8.

Smeets, K., Ruytinx, J., Van Belleghem, F., Semane, B., Lin, D., Vangronsveld, J. and Cuypers, A. (2008b) Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. *Plant Physiol Biochem*, **46**, 212–218.

Smeets, K., Opdenakker, K., Remans, T., Van Sanden, S., Van Belleghem, F., Semane, B., Horemans, N., Guisez, Y., Vangronsveld, J. and Cuypers, A. (2009) Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. *J Plant Physiol*, **166**, 1982–1992.

Srivastava, A. and Jaiswal, V.S. (1989) Biochemical changes in duck weed after cadmium treatment. Enhancement in senescence. *Water, Air, Soil Pollut*, **50**, 163–170.

Suzuki, N. (2005) Alleviation by calcium of cadmium-induced root growth inhibition in *Arabidopsis* seedlings. *Plant Biotechnol*, **22**, 19–25.

Van Belleghem, F., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, J., d'Haen, J. and Valcke R. (2007) Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytol*, **173**, 495–508.

CHAPTER 4

Alternative respiration as a primary defence during cadmium-induced mitochondrial oxidative challenge in *Arabidopsis thaliana*

Els Keunen, Marijke Jozefczak, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2013) Alternative respiration as a primary defence during cadmium-induced mitochondrial oxidative challenge in *Arabidopsis thaliana*. Environmental and Experimental Botany, 91, 63–73.

ABSTRACT

Plant growth and development can be highly restricted by environmental stressors such as cadmium (Cd) pollution. The mitochondrial non-phosphorylating alternative respiratory pathway, mediated by alternative oxidase (AOX), alternative NAD(P)H dehydrogenases (NDs) and uncoupling protein (UCP), was suggested to be crucial in the acclimation of plants to fluctuating environmental conditions. Therefore, we examined the effects of environmentally realistic Cd exposure (5 and 10 μM) on the alternative respiratory chain in *Arabidopsis thaliana* using a kinetic exposure setup. We demonstrated that during exposure to Cd, *Arabidopsis* seedlings show a mitochondrial oxidative challenge to which they acutely respond by increasing the transcript level of several *AOX*, *ND* and *UCP* isoforms in both roots and leaves. In addition, AOX protein levels increased during acute Cd exposure (2 and 24 h). Based on our data, we suggest the formation of a condensed non-phosphorylating electron transport chain (ETC) functioning through cytosolic NDs and AOX, with co-regulation of *ND* and *AOX* expression during Cd stress. Therefore, both enzymes might cooperate in the potential acclimation of *Arabidopsis* seedlings to environmentally realistic Cd exposure by modulating the extent of mitochondrial ROS production.

Keywords:

alternative respiration, *Arabidopsis thaliana*, cadmium, gene families, mitochondria, oxidative stress.

4.1. INTRODUCTION

Many regions worldwide have to cope with soil metal pollution representing a major adverse environmental stress factor. Since the past century, metals were spread into the environment due to industrial emissions and the use of fertilisers, pesticides or sewage sludge in agriculture. Contamination with the non-essential element cadmium (Cd), even at trace concentrations, is of great concern because of its potential threats for organisms. In plants, Cd negatively affects growth, physiological and biochemical processes such as photosynthesis and transpiration (DalCorso *et al*. 2008, Cuypers *et al*. 2009). In addition, Cd uptake by crops represents its main entry route into the food chain, causing important concerns for human health (Nawrot *et al*. 2006, Cuypers *et al*. 2010, Gallego *et al*. 2012).

Although Cd is a non-redox-active metal, its phytotoxicity is related to the generation of reactive oxygen species (ROS) leading to oxidative stress (Cuypers *et al*. 2012 and references therein). Although this may result in cellular damage, ROS also initiate various signalling pathways leading to acclimation. Plant mitochondria are important players in the cellular redox homeostasis and signalling at the crossroads of life versus death (Noctor *et al*. 2007). Under standard physiological conditions, ROS are continuously produced as metabolic by-products at the level of the mitochondrial electron transport chain (ETC). The plant mitochondrial ETC is also considered to be a major target of Cd toxicity, as it was shown to be the primary site of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) production in 10 or 30 µM Cd-exposed *Solanum tuberosum* tuber mitochondria (Heyno *et al*. 2008). As shown by Schwarzländer *et al*. (2009), mitochondria are highly sensitive to Cd-provoked redox disturbances, with a slowly recovering redox state after an oxidative insult. Therefore, plant mitochondria could be involved in both perception and response signalling mechanisms during the oxidative challenge mediated by Cd exposure in plants.

In addition to antioxidative ROS scavenging enzymes and metabolites (Navrot *et al*. 2007), various energy-dissipating but ROS-mediating alternative respiratory components such as the alternative oxidase (AOX; McDonald 2008), type II alternative NAD(P)H dehydrogenases (NDs; Rasmusson *et al*. 2008) and uncoupling protein (UCP; Vercesi *et al*. 2006) are present in plant mitochondria.

Research over the years implied their importance in the acclimation of plants to fluctuating environmental surroundings, as their expression and activities were intensively enhanced by drought (Bartoli *et al*. 2005), high light (Yoshida *et al*. 2011), salinity (Smith *et al*. 2009) and other abiotic stress factors. In addition, both AOX (Maxwell *et al*. 1999) and UCP (Pastore *et al*. 2007) reduce the risk of mitochondrial ROS formation. Recently, Cvetkovska and Vanlerberghe (2012) established for the first time that a lack of AOX increases steady-state *in planta* mitochondrial $O_2^{\bullet-}$ concentrations in tobacco leaves, which exemplifies the potential importance of AOX during a Cd-induced oxidative challenge at the mitochondrial level (Keunen *et al*. 2011a and references therein).

The involvement of AOX in Cd stress responses was demonstrated before in the protist *Euglena gracilis*, with an increased AOX content and capacity related to the protist's Cd resistance mechanism (Castro-Guerrero *et al*. 2008). Similarly, Duan *et al*. (2010) suggested AOX as an indicator of a plant's resistance to Cd based on its differential response in three wheat cultivars. In addition, Garmash and Golovko (2009) have shown strong stimulatory effects of Cd concentrations ranging from 30 to 100 µM on the alternative respiratory rate and assigned a major homeostatic role to this pathway in Cd-stressed barley plants. Recently, Wang *et al*. (2013) have demonstrated that Cd-induced alternative respiration is an important detoxification mechanism in rice. The emerging involvement of AOX and thus alternative respiration in metabolic homeostasis and (abiotic) stress signalling pathways originating from the mitochondrion was recently reviewed by Vanlerberghe *et al*. (2009).

Whereas previous research results support a potential role for AOX and alternative respiration in mediating the responses to highly toxic Cd levels (cfr. *supra*), information whether this enzyme is also involved when the applied Cd concentrations are situated in an environmentally realistic and sublethal range is limited. Finkemeier *et al*. (2005) have shown that alternative respiration of *Arabidopsis thaliana* roots exposed to 10 µM $CdCl_2$ for one week increased up to 40% as compared to 20% under control conditions. However, Cd-induced responses of other components of the alternative respiratory pathway such as NDs and UCP have not been studied before. All enzymes functioning in alternative respiration are nuclear-encoded by small multigene families. At present, an extensive study on how Cd affects all members of the *AOX*, *ND* and

UCP gene families in *A. thaliana* is still lacking. Nevertheless, this information could be of high value to understand their potential role in acclimation responses to Cd exposure in plants. Therefore, the aim of the current study is to reveal the potential link between Cd-induced oxidative stress and both the mitochondrial antioxidative and alternative respiratory pathways at different cellular functional levels. As opposed to most of the previous research, Cd was applied in a moderate and environmentally realistic concentration range as *A. thaliana* seedlings were exposed to either 5 or 10 µM $CdSO_4$ via the roots. Both concentrations are based on the Cd levels measured in the pore water of sandy soils in specific metal polluted Belgian regions (Krznaric *et al*. 2009) and were demonstrated to be sublethal for *A. thaliana* seedlings (Keunen *et al*. 2011b).
To study the sequence of events and responses that are spatially and temporally activated by Cd, both root and shoot samples taken at regular intervals (2, 24, 48 and 72 h after the start of the exposure) were examined. To our knowledge, a detailed transcriptional study of mitochondrial antioxidative and alternative respiratory components dissecting the responses in different organs and at different points in time is still lacking and therefore of high value in studying Cd exposure in plants.

4.2. MATERIALS AND METHODS

4.2.1. Plant culture and cadmium exposure

Wildtype *A. thaliana* seeds (Columbia ecotype) were surface-sterilised and seedlings were grown on hydroponics as described by Smeets *et al*. (2008a) except that purified sand was used instead of rock wool (Keunen *et al*. 2011b). A modified Hoagland nutrient solution was used (section 3.2.1., Smeets *et al*. 2008a) and growth conditions were set at a 12 h photoperiod, 65% relative humidity and day/night temperatures of 22 °C and 18 °C respectively. Light was provided by Philips Green-Power LED modules. A combination of blue, red and far-red modules was used to obtain a spectrum simulating the photosynthetic active radiation (PAR) of sunlight. The PAR provided at the rosette level was 170 µmol m^{-2} s^{-1}. After 20 days of growth, control samples (0 h) were taken prior to exposing the plants to $CdSO_4$ (0, 5 or 10 µM) supplied to the roots. After 2, 24, 48 and 72 h, root and leaf (entire shoot) samples were taken, snap frozen

in liquid nitrogen and stored at -70 °C for further analyses, except for element analysis (cfr. *infra*). During sampling, root and leaf biological replicates were harvested from – depending on the required sample weight – one or more seedlings out of one pot at a given point in time. To avoid within-pot correlation (Smeets *et al*. 2008a), different biological replicates were sampled out of other pots containing the same Cd concentration.

4.2.2. Element analysis

During harvest, roots were washed for 15 min with 10 mM $Pb(NO_3)_2$ at 4 °C to exchange surface-bound metals and rinsed in distilled water (Cuypers *et al*. 2002), while leaves were only rinsed in distilled water. Samples were oven-dried and digested with 70-71% HNO_3 in a heat block. Next to Cd, concentrations of macronutrients (Mg, Ca, K, Na, P and S) and micronutrients (Zn, Cu, Mn and Fe) were determined via inductively coupled plasma – atomic emission spectrometry (ICP-AES, Perkin-Elmer, 1100B, USA). For reference purposes, blank (HNO_3 only) and standard [NIST Spinach (1570a)] samples were used.

4.2.3. Lipid peroxidation analysis

As a measure of lipid peroxidation, the amount of thiobarbituric acid (TBA) reactive metabolites in root and leaf samples was determined spectrophotometrically. Plant tissue was homogenised in 0.1% trichloroacetic acid (TCA), centrifuged (10 min, 20 000 *g*, 4 °C) and diluted in 0.5% TBA. Blank samples (0.1% TCA only) were used as a reference. After heating the extracts at 95 °C during 30 min, they were briefly cooled on ice-water and centrifuged for 10 min (20 000 *g*, 4 °C). The absorbance of the supernatant was measured at 532 nm and corrected for unspecific absorbance at 600 nm (ε = 155 mM^{-1} cm^{-1}).

4.2.4. Gene expression analysis

Frozen root and leaf tissues were disrupted in 2 mL microcentrifuge tubes under frozen conditions using two stainless steel beads and the Retsch Mixer Mill MM2000 (Retsch, Haan, Germany). From the disrupted tissues, RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion, Applied Biosystems, Foster City, CA, USA). The RNA concentration and purity of the samples was assessed spectrophotometrically on the NanoDrop® ND-1000 (ThermoScientific,

Wilmington, DE, USA). To eliminate genomic DNA contamination, the extracted RNA was subjected to a DNase treatment using the TURBO DNA-*free*™ Kit (Ambion, Applied Biosystems, Foster City, CA, USA). The treated RNA was converted to single stranded cDNA via the High-Capacity cDNA Reverse Transcription Kit (Ambion, Applied Biosystems, Foster City, CA, USA), where equal RNA amounts (1 µg) were present in all samples. A tenfold dilution of the cDNA was made in 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0; Sigma-Aldrich, Belgium) and stored at -20 °C.

Quantitative real-time PCR was performed in optical 96-well plates using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and SYBR Green chemistry. Gene-specific forward and reverse primers (300 nM) were designed and optimised via the Primer Express software (v2.0, Applied Biosystems, Foster City, CA, USA). Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C) followed by the generation of a dissociation curve to verify amplification specificity. Reactions contained 2 µL diluted cDNA template (or RNase free H_2O for the 'no template controls'), 5 µL 2x Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), forward and reverse primers (0.3 µL each) and 2.4 µL RNase-free H_2O in a total volume of 10 µL.

The specificity of the used primer pairs was checked *in silico* using Blast (http://www.arabidopsis.org/Blast/index.jsp) and after qPCR by verifying single peaks on the dissociation curve. In addition, primer efficiency was verified to be higher than 80%. In Supplementary Table 4.1, all gene annotations and primer sequences are shown.

Gene expression levels were calculated according to the $2^{-\Delta Cq}$ method relative to the sample with the highest expression (minimum Cq). The data obtained were normalised to the expression of three stable reference genes selected out of a set of 10 (Remans *et al*. 2008) by geNorm (v3.5, Vandesompele *et al*. 2002) and Normfinder (v0.953, Andersen *et al*. 2004) algorithms. For the roots, data were normalised using the geometric average of the $2^{-\Delta Cq}$ values for *AT4G34270* (TIP41-like), *AT5G55840* (PPR gene) and *AT3G18780* (ACT2), while for the leaves *AT5G25760* (UBC), *AT2G28390* (SAND family) and *AT4G34270* (TIP41-like) were the most stable reference genes used to determine sample-specific normalisation factors.

4.2.5. Hierarchical clustering of gene expression data

To identify genes that are co-expressed during the kinetic Cd exposure, a hierarchical clustering analysis was performed using GenEx software (v4.3.1, MultiD Analyses AB, Göteborg, Sweden). This analysis is based on four different algorithms (unweighted pairs, single and complete linkage and Ward's algorithm), while distances between the measures were calculated via the Euclidian Distance Measure.

4.2.6. The analysis of gene families

To calculate the relative abundance of distinct gene family members, the expression level of each family member was determined for the control sample panel (0 h, 0 µM Cd) relative to the lowest expressed family member. This gives rise to a relative abundance factor for each member of the gene family, which is used in the calculation of its relative abundance in the time-course Cd exposure experimental setup. To be able to compare expression levels between roots and leaves, data were normalised using the geometric average of the $2^{-\Delta Cq}$ values for *AT4G34270* (TIP41-like), *AT5G55840* (PPR gene) and *AT3G18780* (ACT2), the most stable reference genes in both root and leaf control samples as determined by geNorm and Normfinder algorithms. Next, the changes in expression level for each member of a gene family were determined in function of the exposure time and Cd concentration applied and set relatively to the control (0 h, 0 µM Cd).

4.2.7. AOX protein analysis

Protein extractions from whole root and leaf tissues (100 mg fresh weight) were performed as described by Martínez-García *et al*. (1999). Total protein content was quantified using the colorimetric Bio-Rad *DC* Protein Assay (Bio-Rad Laboratories, Nazareth, Belgium). Equal amounts of extracted proteins (35 µg for root and 50 µg for leaf samples) were loaded for all samples and separated by 4-12% SDS-PAGE under standardised running conditions. For immunoblotting purposes, proteins were transferred to a 0.45 µm nitrocellulose membrane according to the manufacturer's protocol. Immunodetection of AOX protein was achieved by the monoclonal AOA antibody, which was kindly provided by Dr. Thomas Elthon (Elthon *et al*. 1989). After blocking with Phosphate Buffered Saline Tween-20 (PBST), blots were incubated with the primary antibody at a

1:100 dilution for 1 h at room temperature. After several wash steps with PBST, blots were incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (ThermoScientific Pierce, Erembodegem-Aalst, Belgium) at a 1:1500 dilution for 1 h at room temperature. Chemiluminescence was visualised using the Pierce® ECL Western Blotting Substrate (ThermoScientific Pierce, Erembodegem-Aalst, Belgium) and the ImageQuant-RT ECL device (GE Healthcare, Diegem, Belgium). Experiments were repeated at least twice using independent biological replicates and representative results are shown.

4.2.8. Statistical analysis

The datasets were analysed via the General Linear Models (GLM) procedure in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Both normality and homoscedasticity were checked; transformations were applied when necessary to approximate normality. The Tukey-Kramer adjustment for multiple comparisons was applied to obtain corrected p-values.

4.3. RESULTS

4.3.1. Growth, Cd and element uptake

To study the effects of environmentally realistic Cd concentrations (5 and 10 µM) on plant growth, the fresh weight of both roots and leaves was determined at five harvest time points (Figure 4.1). Cadmium obviously has a dose-dependent effect on *Arabidopsis* root growth, with complete inhibition in case of 10 µM Cd exposure (Figure 4.1A). However, plants exposed to 5 µM Cd had a continuous higher root biomass production during the time of exposure as compared to 10 µM Cd-exposed plants (Figure 4.1A). The dose-dependent effect of Cd on plant growth was also observed for the leaves with no increase in leaf fresh weight for plants exposed to the highest Cd concentration (Figure 4.1B), but a continuous increase for 5 µM Cd-exposed plants.

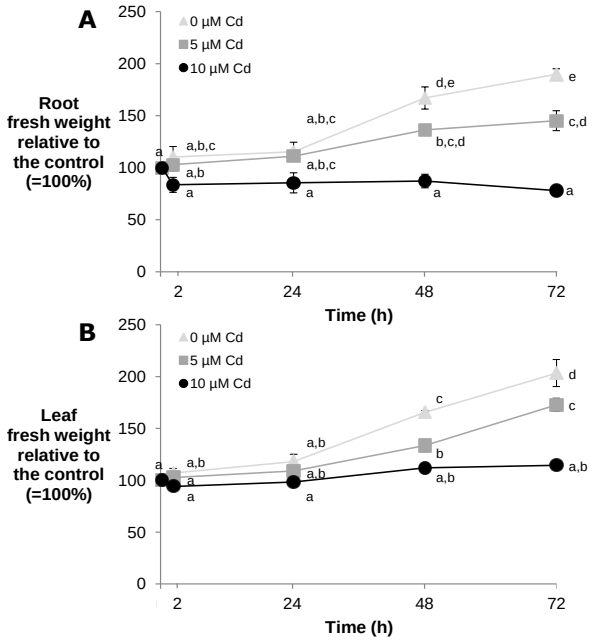
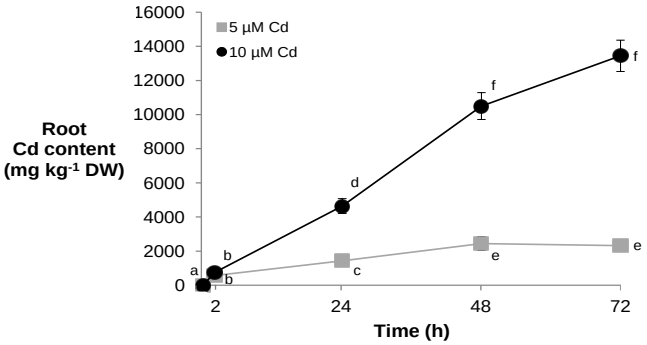


Figure 4.1. *Exposure to Cd has an effect on the growth of Arabidopsis thaliana plants. Plants were exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 3 biological replicates relative to the control (0 h, 0 µM) set at 100%. (A) Fresh root weight. The letters a-e represent groups with a significantly different fresh weight ($p < 0.05$). (B) Fresh leaf weight. The letters a-d represent groups with a significantly different fresh weight ($p < 0.05$).*

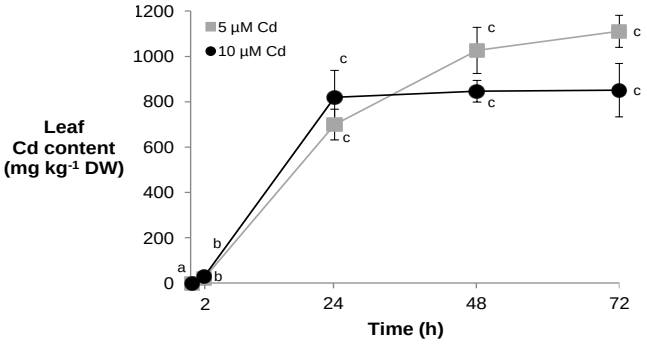
The uptake of Cd by the plants was verified by determining its content in dried roots and leaves (Figure 4.2). The root Cd content not only increased over time, but also with the dose (Figure 4.2A). In the leaves however, such a dose-dependent increase was not observed, with the maximum Cd content already reached at 24 h for both concentrations (Figure 4.2B). The Cd root-to-leaf translocation in plants exposed to 5 µM Cd was relatively constant during the exposure period from 24 h on. However, plants exposed to the highest Cd concentration showed a decreased Cd translocation over time and never reached a similarly high rate (Figure 4.2C).

To get an indication of how Cd exposure influenced nutrient acquisition in roots and leaves, the content of macronutrients (Mg, Ca, K, Na, P and S) and micronutrients (Zn, Cu and Mn and Fe) was determined. Remarkably, the Ca content significantly increased in the roots of plants exposed to the highest Cd concentration (Figure 4.2D), which was not observed in the leaves. Alterations in the amounts of other nutrients were limited in both organs (data not shown).

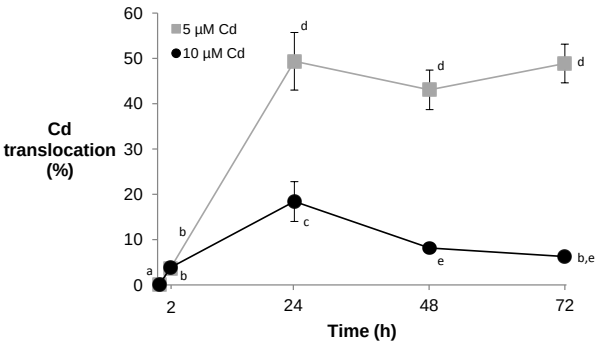
A


Root
Cd content
(mg kg-1 DW)
5 µM Cd
10 µM Cd
Time (h)

B


Leaf
Cd content
(mg kg-1 DW)
5 µM Cd
10 µM Cd
Time (h)

C


Cd
translocation
(%)
5 µM Cd
10 µM Cd
Time (h)

D

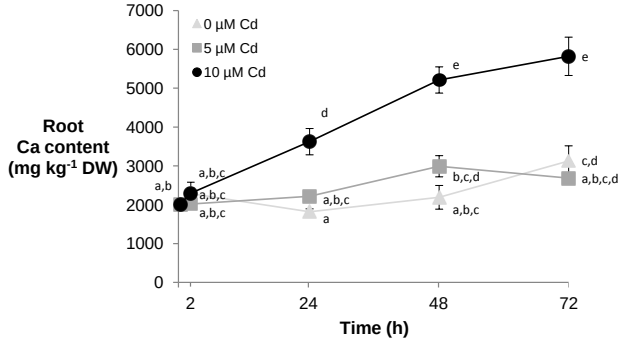

Root
Ca content
(mg kg-1 DW)
0 µM Cd
5 µM Cd
10 µM Cd
Time (h)

Figure 4.2. *Element concentrations in roots and leaves of Arabidopsis thaliana plants exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 3 biological replicates. (A) Cadmium content in mg kg^{-1} dry weight (DW) in the roots of Cd-exposed plants. No Cd could be detected in unexposed roots. The letters a-f represent groups with a significantly different Cd content ($p < 0.05$). (B) Cadmium content in mg kg^{-1} dry weight (DW) in the leaves of Cd-exposed plants. No Cd could be detected in unexposed leaves. The letters a-c represent groups with a significantly different Cd content ($p < 0.05$). (C) The percentage of Cd translocated from roots to leaves for plants exposed to both Cd concentrations. The letters a-e represent groups with a significantly different translocation percentage ($p < 0.05$). (D) Calcium (Ca) content in mg kg^{-1} dry weight (DW) in the roots. The letters a-e represent groups with a significantly different Ca content ($p < 0.05$).*

4.3.2. A Cd-induced oxidative challenge at cellular and mitochondrial levels

In previous experiments, exposure to 5 and 10 µM Cd during 24 h significantly increased H_2O_2 contents in *Arabidopsis* roots and leaves (Cuypers *et al*. 2011). Although this ROS accumulation can contribute to metal-induced signalling and acclimation responses, it can also lead to oxidative damage. To estimate the extent of membrane damage during prolonged exposure to both Cd concentrations, the amount of TBA reactive metabolites was determined in roots and leaves (Figure 4.3). In roots, exposure to 10 µM Cd significantly increased lipid peroxidation relative to the control at 72 h (Figure 4.3A), while the overall treatment effect was highly significant ($p = 0.0035$). Similar results were obtained for the leaves, where the amount of TBA reactive metabolites only increased in the same condition (Figure 4.3B). However, the overall treatment effect was again highly significant ($p < 0.0001$).

In addition to this Cd-induced oxidative challenge at the cellular level (lipid peroxidation, Figure 4.3, Cuypers *et al*. 2011), we estimated the extent of mitochondrial oxidative stress by measuring the transcript level of a gene termed 'upregulated by oxidative stress' (*UPOX*, *AT2G21640*). This protein is localised in mitochondria (Sweetlove *et al*. 2002, Van Aken *et al*. 2009) and defined as a hallmark of oxidative stress in *Arabidopsis* (Gadjev *et al*. 2006). During our kinetic Cd exposure, its transcription levels were highly upregulated in a dose-dependent manner in both roots and leaves (Table 4.1). To investigate whether this Cd-induced oxidative challenge is counteracted by mitochondrial antioxidative enzymes, transcript levels of their genes were analysed. Although manganese superoxide dismutase (Mn-SOD) is the only mitochondrial SOD

isoform, *MSD1* transcription remained unaltered during Cd exposure in both roots and leaves (Table 4.1). Moreover, none of the other measured antioxidative genes were upregulated by Cd in the roots (Supplementary Table 4.2). Transcript levels of mitochondrial ascorbate peroxidase (*APX*), glutathione reductase (*GR*), monodehydroascorbate reductase (*MDHAR*) and peroxiredoxin (*Prx*) were even downregulated after 72 h (Supplementary Table 4.2). In the leaves, only mitochondrial *DHAR* transcript levels were upregulated at the highest concentration from 24 h after the start of the treatment. Gene expression levels of other mitochondrial antioxidative enzymes were altered in a transient way (Supplementary Table 4.2). These results suggest the presence of alternative mechanisms to counteract the Cd-induced oxidative challenge at the mitochondrial level.

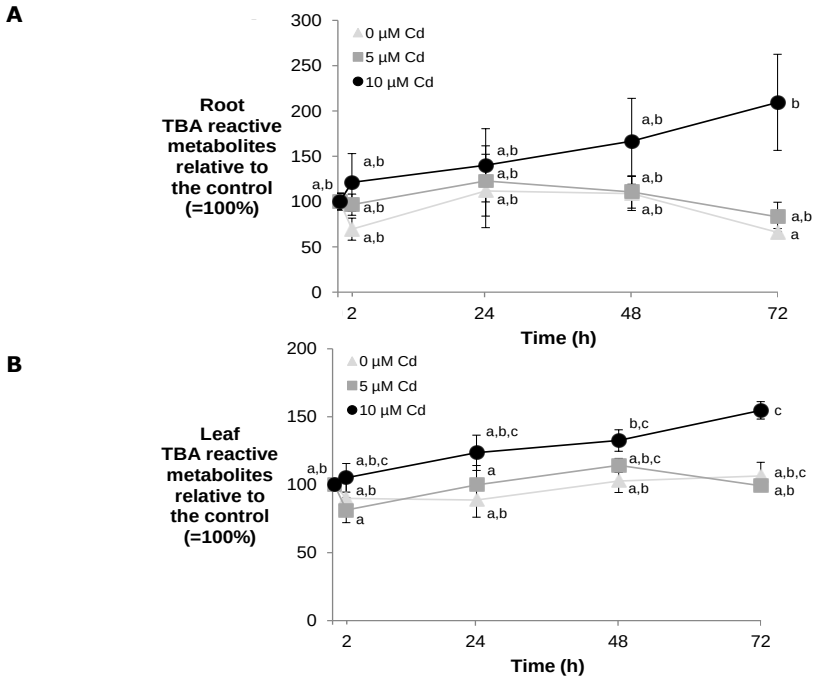


Figure 4.3. *Exposure to Cd leads to lipid peroxidation in roots and leaves of Arabidopsis thaliana plants. Plants were exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of at least 4 biological replicates relative to the control (0 h, 0 µM) set at 100%. (A) The amount of TBA reactive metabolites in the roots. The letters a-b represent groups with a significantly different amount of TBA reactive metabolites ($p < 0.05$). (B) The amount of TBA reactive metabolites in the leaves. The letters a-c represent groups with a significantly different amount of TBA reactive metabolites ($p < 0.05$).*

4.3.3. The response of mitochondrial alternative respiration to Cd exposure is regulated at both transcript and protein levels

The expression patterns of selected nuclear genes encoding several subunits of the mitochondrial phosphorylating respiratory pathway were determined as described by Yoshida and Noguchi (2009) and Watanabe *et al*. (2010). No significant effects were observed in the roots (Supplementary Table 4.3). In the leaves, succinate dehydrogenase 2-1 (*SDH2-1*) expression increased from 24 h after the start of the exposure to both Cd concentrations, while the expression of the 5b subunit of cytochrome c oxidase (complex IV) (*COX5b*) significantly decreased after 72 h exposure to 10 µM Cd (Supplementary Table 4.3).

Transcriptional upregulation of the non-phosphorylating AOX, ND and UCP enzymes has shown to occur during various stress conditions. In eudicots, AOXs are nuclear-encoded by a small multigene family consisting of *AOX1* and *AOX2* subfamilies. As *AOX2* expression is restricted to the mature seed (Clifton *et al*. 2006) and constitutive or developmentally regulated (Polidoros *et al*. 2009), this gene was omitted from our analysis. In *Arabidopsis*, four genes belong to the stress-responsive *AOX1* subfamily (*AOX1a*, *AOX1b*, *AOX1c* and *AOX1d*). In the roots, only *AOX1a* and *AOX1c* expression could reliably be detected. Both genes were differentially influenced by Cd, without significant alterations in *AOX1c* expression levels (Supplementary Table 4.4A). On the other hand, *AOX1a* transcription was induced in a dose-dependent way after 24 and 48 h of exposure to both 5 and 10 µM Cd. This induction was only significantly sustained at 72 h in roots of plants exposed to the highest Cd concentration, which was similar for the leaves (Table 4.1). However, while *AOX1d* could not accurately be measured in the roots, its expression was dose-dependently induced to a great extent in the leaves (Table 4.1).

Table 4.1. *Exposure to Cd upregulates the expression of genes encoding a mitochondrial marker protein for oxidative stress, alternative oxidase and alternative NAD(P)H dehydrogenase isoforms in Arabidopsis thaliana roots and leaves. Analyses were performed using quantitative real-time PCR. Transcript levels were determined in roots and leaves of A. thaliana plants exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 4 biological replicates relative to the control (0 µM) at each time point set at 1.00. Significance levels relative to the control: $p < 0.05$; $p < 0.01$. UPOX, upregulated by oxidative stress; MSD, manganese superoxide dismutase; AOX, alternative oxidase; ND, alternative NAD(P)H dehydrogenase.*

Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
ROOTS						
UPOX	0	1.00 ± 0.13	1.00 ± 0.15	1.00 ± 0.20	1.00 ± 0.03	1.00 ± 0.09
	5		0.99 ± 0.23	2.71 ± 0.32	3.88 ± 1.34	2.56 ± 0.41
	10		1.26 ± 0.36	4.79 ± 0.52	9.58 ± 2.08	9.25 ± 2.57
MSD1	0	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.05	1.00 ± 0.04
	5		1.05 ± 0.07	1.10 ± 0.09	0.99 ± 0.13	1.06 ± 0.14
	10		0.92 ± 0.06	1.27 ± 0.10	1.20 ± 0.04	1.14 ± 0.06
AOX1a	0	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.12	1.00 ± 0.06	1.00 ± 0.05
	5		1.81 ± 0.27	2.75 ± 0.36	2.47 ± 0.52	1.34 ± 0.19
	10		1.71 ± 0.22	5.12 ± 1.12	7.34 ± 0.66	5.11 ± 0.91
NDA2	0	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.07
	5		0.90 ± 0.09	1.77 ± 0.25	1.42 ± 0.11	1.29 ± 0.19
	10		0.87 ± 0.12	4.47 ± 0.68	4.02 ± 0.38	5.81 ± 0.13
NDB2	0	1.00 ± 0.06	1.00 ± 0.11	1.00 ± 0.24	1.00 ± 0.19	1.00 ± 0.07
	5		1.28 ± 0.12	1.64 ± 0.29	1.64 ± 0.23	0.99 ± 0.25
	10		1.11 ± 0.25	2.29 ± 0.36	3.81 ± 0.12	2.23 ± 0.03
NDB4	0	1.00 ± 0.17	1.00 ± 0.16	1.00 ± 0.30	1.00 ± 0.30	1.00 ± 0.20
	5		1.05 ± 0.23	5.12 ± 0.98	8.84 ± 4.54	2.03 ± 0.41
	10		1.10 ± 0.54	13.64 ± 5.12	7.64 ± 3.45	5.84 ± 1.52

Table 4.1. *Continued.*

Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
LEAVES						
UPOX	0	1.00 ± 0.11	1.00 ± 0.27	1.00 ± 0.06	1.00 ± 0.37	1.00 ± 0.10
	5		1.17 ± 0.10	4.94 ± 1.25	3.74 ± 0.68	3.92 ± 0.99
	10		1.44 ± 0.32	22.45 ± 6.48	6.95 ± 2.21	6.68 ± 2.15
MSD1	0	1.00 ± 0.03	1.00 ± 0.12	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.05
	5		1.21 ± 0.05	0.90 ± 0.08	1.09 ± 0.03	1.14 ± 0.09
	10		1.24 ± 0.14	1.28 ± 0.10	1.29 ± 0.08	1.21 ± 0.09
AOX1a	0	1.00 ± 0.06	1.00 ± 0.17	1.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.10
	5		0.96 ± 0.11	7.76 ± 0.28	3.37 ± 0.43	2.40 ± 0.52
	10		1.13 ± 0.11	11.85 ± 2.57	7.55 ± 1.43	5.74 ± 1.57
AOX1d	0	1.00 ± 0.81	1.00 ± 0.67	1.00 ± 0.38	1.00 ± 0.66	1.00 ± 0.32
	5		2.54 ± 1.77	2804.08 ± 965.74	83.36 ± 25.27	203.68 ± 94.97
	10		16.92 ± 9.08	5838.49 ± 1247.11	302.70 ± 78.58	693.16 ± 190.44
NDA2	0	1.00 ± 0.05	1.00 ± 0.38	1.00 ± 0.10	1.00 ± 0.28	1.00 ± 0.03
	5		0.82 ± 0.05	14.10 ± 3.27	5.12 ± 0.09	3.54 ± 0.37
	10		1.10 ± 0.15	23.50 ± 4.37	7.50 ± 0.94	6.95 ± 0.79
NDB2	0	1.00 ± 0.06	1.00 ± 0.36	1.00 ± 0.05	1.00 ± 0.25	1.00 ± 0.01
	5		0.73 ± 0.06	11.07 ± 1.77	3.61 ± 0.08	2.63 ± 0.26
	10		1.06 ± 0.16	17.28 ± 1.32	5.03 ± 0.42	3.86 ± 0.57
NDB4	0	1.00 ± 0.16	1.00 ± 0.35	1.00 ± 0.25	1.00 ± 0.51	1.00 ± 0.20
	5		0.30 ± 0.04	6.77 ± 2.40	15.14 ± 4.00	19.61 ± 3.63
	10		0.81 ± 0.25	10.61 ± 2.82	10.50 ± 2.06	11.22 ± 4.12

In addition to AOX, the plant mitochondrial respiratory chain contains alternative NDs at the inner (internal NDs) and outer mitochondrial membrane (external NDs), which bypass respiratory complex I. In *A. thaliana*, seven nuclear genes encode alternative NDs grouped into internal (*NDA1*, *NDA2* and *NDC1*) and external (*NDB1*, *NDB2*, *NDB3* and *NDB4*) isoforms (Rasmusson *et al*. 2008). In our analysis, *NDB3* expression could not be detected in roots and did not show any significant alterations during Cd exposure in the leaves (Supplementary Table 4.4B). However, the expression of *NDA2*, *NDB2* and *NDB4* did show significant increases in both organs of Cd-exposed seedlings (Table 4.1). In roots and leaves exposed to the highest Cd concentration, the expression of *NDB1* and *NDC1* decreased significantly. In addition, *NDA1* expression showed a significant decrease for both Cd concentrations at 24 h in the leaves, which was only sustained at 72 h for the highest Cd concentration (Supplementary Table 4.4B).

Several respiratory genes of the *AOX* and *ND* gene families displayed similar expression responses to Cd exposure (Table 4.1). Therefore, we used GenEx for a hierarchical clustering analysis of the Cd-induced expression patterns of all measured mitochondrial antioxidative and respiratory genes for roots and leaves separately. This analysis enables the identification of coordinately regulated genes based on raw gene expression values and revealed co-expression of *AOX1a*, *NDA2* and *NDB2* in the roots (Figure 4.4A). Interestingly, these genes clustered together with *UPOX*, a marker for oxidative stress (Figure 4.4A). In the leaves, *AOX1a* and *AOX1d* showed similarly regulated expression profiles with a cluster of *NDA2* and *NDB2* (Figure 4.4B). Full clusters are shown in Supplementary Figure 4.1.

Uncoupling proteins (UCP) catalyse a proton leak that dissipates the proton electrochemical gradient over the inner mitochondrial membrane, thereby shortcutting the ATP synthase complex and thus oxidative phosphorylation (Vercesi *et al*. 2006). Borecký *et al*. (2006) depicted the genomic structure and expression profiles of six putative members of the *UCP* gene family in *A. thaliana*. However, *UCP6* expression could not be detected in any tissue or organ and was suggested to be a pseudogene or a gene expressed in very low levels (Borecký *et al.* 2006). Therefore, we determined the effects of Cd exposure on isoforms *UCP1* to *UCP5*. In roots, a mainly decreasing trend was

observed, which was only significant for *UCP2* and *UCP3* (Supplementary Table 4.4C). In the leaves, exposure to both 5 and 10 µM Cd evoked a significantly reduced *UCP2* expression after 24 h. However, Cd exposure strongly enhanced the expression levels of *UCP4* and *UCP5*, with a peak for both genes at 24 h (Supplementary Table 4.4C).

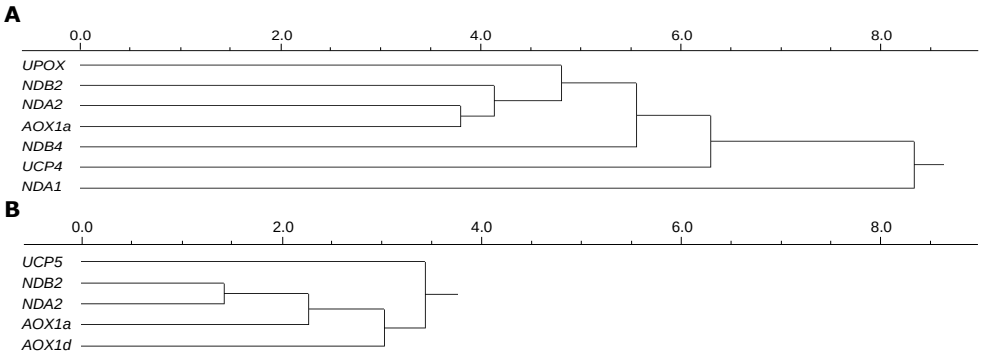


Figure 4.4. *GenEx clusters predicting possible co-regulation of genes encoding alternative respiratory pathway enzymes in roots (A) and leaves (B) of Arabidopsis thaliana plants exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions.*

From the above, it is clear that Cd-induced regulation of the alternative non-phosphorylating respiratory pathway occurs at the level of transcription in both roots and leaves. To verify whether the transcriptional response of AOX to Cd stress was also translated to an increase in protein amount, western blotting was used. In the roots, an immediate (after 2 h) but transient increase in AOX protein levels was observed after exposure to both Cd concentrations (Figure 4.5A). At later time points however, AOX protein levels showed a dose-dependent decrease relative to the control (data not shown). In the leaves, a clear peak in AOX protein amount was detected after 24 h exposure to 10 µM Cd (Figure 4.5B), which again disappeared at later time points (data not shown).

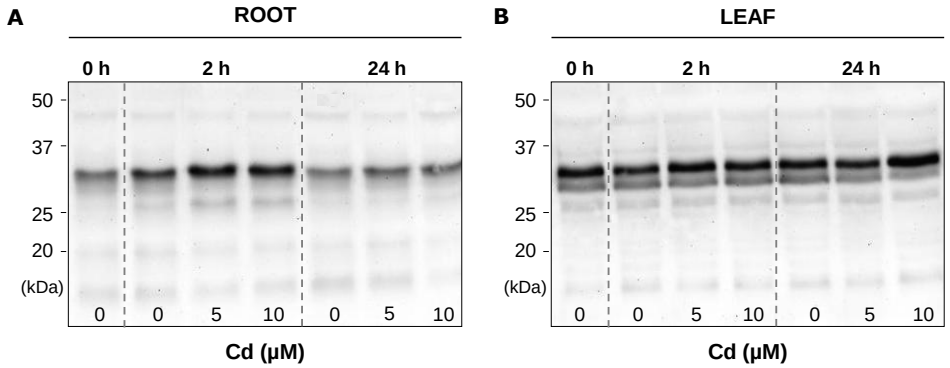


Figure 4.5. *Exposure to Cd leads to a fast but transient increase in AOX protein levels in roots and leaves of Arabidopsis thaliana plants. Plants were exposed to 5 or 10 µM $CdSO_4$ during 2 and 24 h or grown under control conditions. (A) The amount of AOX protein detected via western blotting in the roots. (B) The amount of AOX protein detected via western blotting in the leaves.*

4.3.4. The analysis of mitochondrial alternative respiratory gene families during Cd exposure

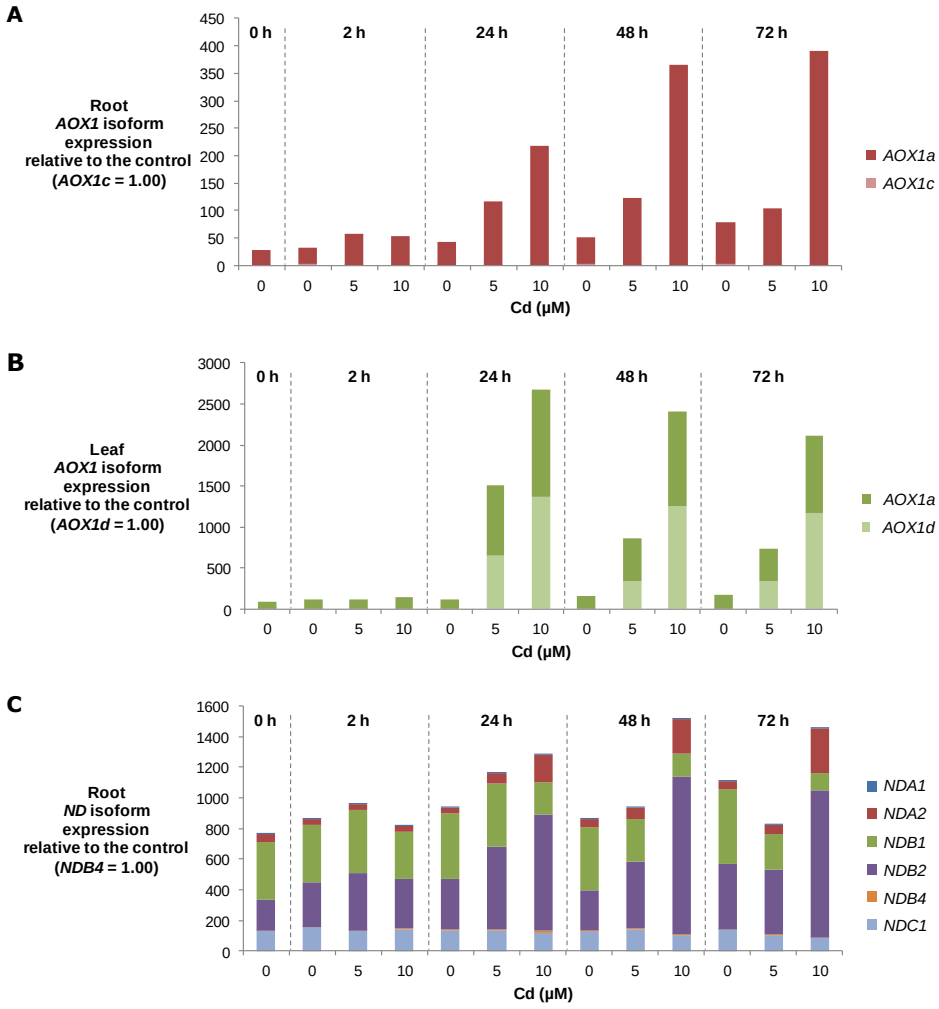
To reveal which members of the *AOX*, *ND* and *UCP* gene families prevail during the kinetic Cd exposure in both roots and leaves, we determined their abundance relative to the control (0 h, 0 µM), with the abundance of the lowest expressed family member set at 1.00.

In the roots, *AOX1a* is the dominant isoform in both control and Cd conditions as shown in Figure 4.6A. In addition, the dose-dependent increase of this isoform is demonstrated after 24, 48 and 72 h (Figure 4.6A). Whereas *AOX1a* is also the most highly expressed in unexposed leaves, *AOX1d* expression catches up with *AOX1a* from 24 h of exposure to both Cd concentrations (Figure 4.6B).

In control conditions, the external isoforms *NDB1* and *NDB2* dominate in the roots (Figure 4.6C). During Cd exposure, the external isoform *NDB2* – and *NDA2* to a lesser extent – becomes prominent with dose-dependent increases in expression after 24, 48 and 72 h. Although *NDB4* transcripts highly increased as shown in Table 4.1, the abundance of this isoform remained rather low as compared to the others (Figure 4.6C). In contrast with the roots, the internal isoforms *NDA1* and *NDC1* were the most abundant under control conditions in the leaves. However, a shift to the external *NDB2* isoform was observed with a clear peak at 24 h exposure to both Cd concentrations (Figure 4.6D). Again, the relative abundance of *NDB4* was lower over the entire time course, although its

transcripts highly increased as shown in Table 4.1. From the above, it is clear that external NDs are important during Cd stress in both organs.

For the *UCP* gene family, *UCP1* and *UCP5* are the most dominant isoforms under control conditions in both roots and leaves (Figure 4.6E and 4.6F). In the roots, no specific isoform prevailed during Cd exposure (Figure 4.6E), while the abundance of *UCP5* was prominent after exposure to both 5 and 10 µM Cd with a clear peak at 24 h (Figure 4.6F).



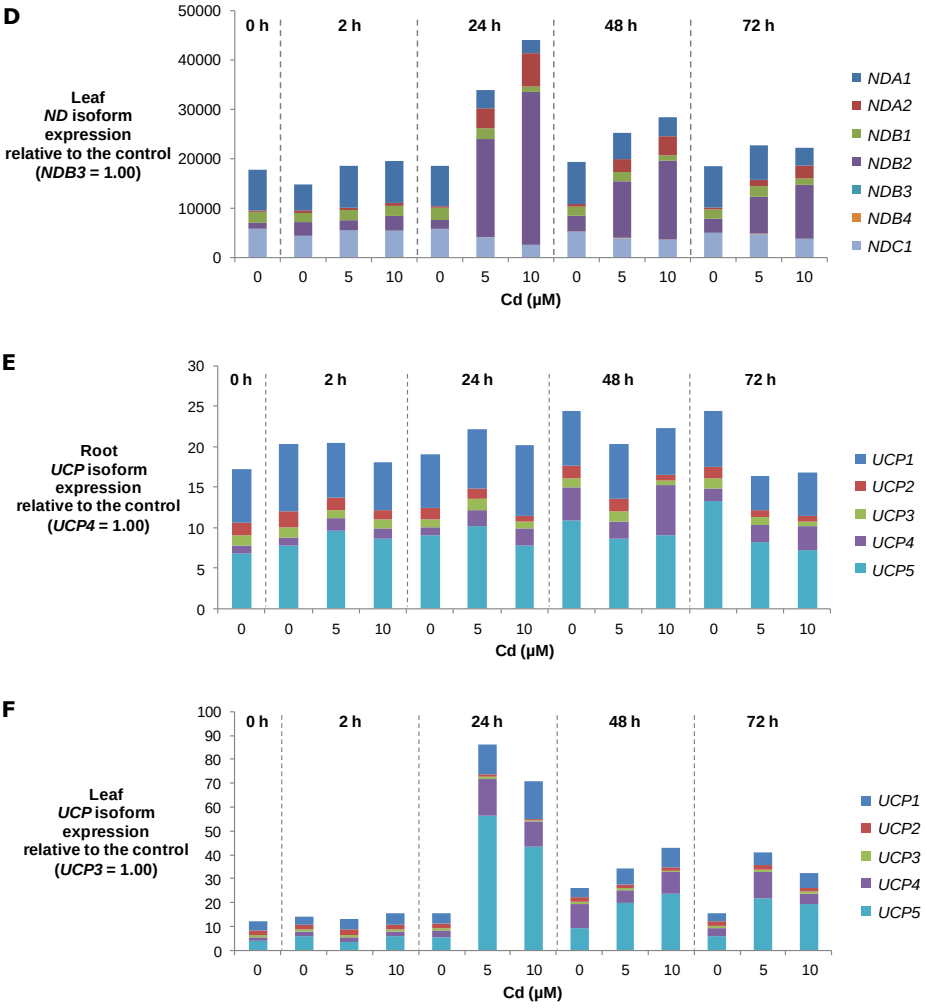


Figure 4.6. *Exposure to Cd has an effect on the relative abundance of AOX, ND and UCP gene family members in roots and leaves of Arabidopsis thaliana plants. Plants were exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean abundance of at least 4 biological replicates relative to the control (0 h, 0 µM) with the abundance of the lowest expressed family member set at 1.00. (A) The relative abundance of AOX1 gene family members in the roots. (B) The relative abundance of AOX1 gene family members in the leaves. (C) The relative abundance of ND gene family members in the roots. (D) The relative abundance of ND gene family members in the leaves. (E) The relative abundance of the UCP gene family members in the roots. (F) The relative abundance of the UCP gene family members in the leaves.*

4.4. DISCUSSION

Although Cd is a non-redox-active metal, it is able to induce oxidative stress (Gallego *et al*. 2012). The connection between metal toxicity and cellular redox disturbances has been the subject of intensive research (Sharma and Dietz 2009) and recently, plant mitochondria were suggested to concurrently be targets and regulators of Cd-induced oxidative stress and resulting signalling (Bi *et al*. 2009). However, as most experiments were carried out using Cd concentrations in a highly toxic range (Garmash and Golovko 2009, Schwarzländer *et al*. 2009), it remains an open question whether Cd concentrations present in soils from contaminated fields provoke similar effects.

4.4.1. Morphological versus biochemical effects of Cd exposure

Earlier range-finding experiments on *A. thaliana* demonstrated that chronic exposure to environmentally realistic Cd concentrations (5 and 10 µM) allows plant survival and reproduction (Keunen *et al*. 2011b). Therefore, the experimental setup of the current study to investigate the acute (2 and 24 h) and prolonged (48 and 72 h) 5 and 10 µM Cd-induced oxidative challenge and its link to mitochondrial pathways in *Arabidopsis* seedlings is justified. The dose-dependent effect of Cd on vegetative plant growth (Keunen *et al*. 2011b) was confirmed for both roots and leaves after 48 and 72 h exposure to both Cd concentrations (Figure 4.1), without significant differences at prior time points in accordance with data reported by Cuypers *et al*. (2011). Elevated external Cd concentrations evoked dose-dependent increases in root Cd contents (Figure 4.2A), which could be related to the inhibition of root growth in this condition (Figure 4.1A). This dose-dependency was not observed in the leaves (Figure 4.2B), which corresponds to the results of Smeets *et al*. (2008b). This could explain the similar effects of both Cd concentrations on regenerative growth as previously observed (Keunen *et al*. 2011b).

However, before morphological disturbances appear, Cd interferes with various biochemical and molecular processes *in planta* (Lagriffoul *et al*. 1998). Exposure to Cd has been linked to an indirect generation of ROS, thereby imposing a cellular oxidative challenge (Cuypers *et al*. 2012). Lipid peroxidation, a clear marker of ROS-induced oxidative damage, was shown to occur under Cd exposure depending on the metal concentration applied (Sandalio *et al*. 2001,

Cuypers *et al*. 2011). However, lipid peroxides may also increase tolerance to forthcoming oxidative insults as recently discussed by Chen and Niki (2011). In the current study, the involvement of oxidative stress was endorsed as an increased lipid peroxidation level in roots and leaves of plants exposed to 10 µM Cd, albeit only significant after 72 h in the roots (Figure 4.3). Corresponding to our data, Collin *et al*. (2008) reported only slightly augmented malondialdehyde levels in leaves of plants exposed to 75 µM $CdSO_4$ for even longer time periods as compared to our experimental setup.

4.4.2. A Cd-induced mitochondrial oxidative challenge – how to counteract?

At the subcellular level, Heyno *et al*. (2008) demonstrated a fast Cd-induced stimulation of ROS generation inside root cells, mainly originating from the mitochondrial ETC. In our experimental setup, it is clear that Cd evokes an oxidative challenge at the level of the mitochondria, as the transcript level of the hallmark protein UPOX is strongly enhanced in both roots and leaves (Table 4.1). Like other cellular compartments, mitochondria possess an extended antioxidative defence system to potentially counteract this oxidative challenge (Navrot *et al*. 2007). Therefore, we determined transcript levels of specific antioxidative defence enzymes functioning in *Arabidopsis* mitochondria. Generally, exposure to Cd had only a minor influence on the expression of their genes. Although Mn-SOD is the only mitochondrial enzyme capable of $O_2^{\bullet-}$ conversion to H_2O_2, its transcript levels did not change under Cd exposure in both roots and leaves (Table 4.1). While Morgan *et al*. (2008) demonstrated that reducing Mn-SOD had a negative impact on the mitochondrial redox state and *Arabidopsis* plant growth, its involvement in counteracting the Cd-induced mitochondrial oxidative challenge was negligible in our setup.

We only detected a significant increase in *Prx* expression in the leaves after 48 h, whereas a decreased transcription was observed for this gene in the roots (Supplementary Table 4.2). Nonetheless, employing T-DNA insertion mutants of *A. thaliana* lacking expression of *AtPrxII F* (KO-*At*PrxII F), Finkemeier *et al*. (2005) assigned a major role to this mitochondrial peroxiredoxin isoform F in antioxidative defence and redox signalling in plant cells. After exposure to

7.5 µM $CdCl_2$, the root growth of these knockout mutants was more limited as compared to wildtype seedlings (Finkemeier *et al*. 2005).

Thioredoxins reduce disulfide bridges of oxidised target proteins, after which they get back-reduced by NADPH-dependent thioredoxin reductases (NTR). In *Arabidopsis*, *NTRA* encodes the major cytosolic isoform, whereas *NTRB* is suggested to express the main mitochondrial isoform (Reichheld *et al*. 2005). Both *NTR* transcripts responded oppositely in Cd-exposed leaves (Supplementary Table 4.2). Interestingly, it was the cytosolic isoform that showed an increasing response rather than the mitochondrial isoform. This coincides with distinct changes in cytosolic antioxidative defence gene expression in *Arabidopsis* exposed to 5 or 10 µM Cd, almost immediately after the onset of the exposure and potentially coping with the cellular oxidative challenge evoked by Cd (Jozefczak M., personal communication). However, to counteract the Cd-induced oxidative challenge in root mitochondria, other mechanisms – next to the mitochondrial antioxidative defence enzymes – must be active, as a general decreasing response is observed here. In the leaves, this might also be the case, as definitely not all measured enzymes are activated at the level of transcription.

4.4.3. An abridged non-phosphorylating ETC is active during acute (24 h) and prolonged Cd stress

One such mechanism might be the alternative respiratory pathway in plant mitochondria, which comprises AOX, NDs and UCP. In the current study, we have shown that upon exposure to Cd, *Arabidopsis* seedlings directly respond by increasing the transcript level of several *AOX*, *ND* and *UCP* isoforms in both roots and leaves (Table 4.1, Supplementary Table 4.4). Moreover, their expression was influenced to a much higher extent as compared to the changes observed for mitochondrial antioxidative and phosphorylating respiratory enzymes in both organs. Therefore, we suggest that upon Cd exposure, phosphorylating respiration might be compromised, which contributes to an increased ROS generation at the mitochondrial level due to electron leakage. However, alternative respiration is then triggered to minimise the extent of ROS production by diverting electrons to AOX reducing O_2.

From the abundance analysis, it is clear that transcriptional inductions peak at 24 h in both roots and leaves (Figure 4.6). The latter observation correlates well with the maximum Cd content reached at 24 h in the leaves (Figure 4.2B). This peak moment might indicate that acutely regulated defence systems are put up to counteract the Cd-induced oxidative challenge, reaching a more equilibrated state after 72 h for plants exposed to 5 µM Cd. However, the dose-dependent manner of transcriptional inductions might indicate that coping with 10 µM Cd requires more time. Indeed, oxidative damage is apparent after 72 h of exposure to this Cd concentration (Figure 4.3) and transcriptional responses are still strongly enhanced. Nonetheless, plants can survive long-term exposure to this concentration as shown by Keunen *et al*. (2011b), in which the alternative respiratory pathway might be involved.

Our results do not assign a major role to UCP and several classical ETC components (Supplementary Tables 4.3 and 4.4C) in consonance with the results of Clifton *et al*. (2005) for a wide range of abiotic stressors. In our study, only *UCP4* and *UCP5* showed increased transcript levels in the leaves (Supplementary Table 4.4C). The best characterised members of the *Arabidopsis UCP* gene family are *UCP1*, *UCP2* and *UCP3*, with the first isoform most abundantly expressed according to Genevestigator (Nogueira *et al*. 2011). This is demonstrated by our gene family analysis under control conditions (Figure 4.6E and 4.6F), where additionally *UCP5* made up an important part. However, Palmieri *et al*. (2008) provided evidence that its gene product – as well as the product of the *UCP4* gene – is a member of the *Arabidopsis* mitochondrial carrier family, transporting dicarboxylic acid across the inner mitochondrial membrane. The increased *UCP4* and *UCP5* transcripts in the leaves of Cd-exposed seedlings (Figure 4.6F) could therefore reflect the enhanced need to exchange respiration and tricarboxylic acid (TCA) cycle substrates in stress conditions as suggested by Van Aken *et al*. (2009).

Based on our data, we suggest the formation of a condensed non-phosphorylating ETC functioning through cytosolic NDs and AOX, as *NDB2*, *NDB4* and *AOX1a* are the most prominently induced isoforms during Cd exposure in both roots and leaves (Table 4.1). Concurrently, Cd exposure evoked a strongly significant induction of *AOX1d* as compared to *AOX1a* in the leaves (respectively 5800-fold versus 12-fold at their peak expression,

Table 4.1). Clifton *et al*. (2006) analysed the expression patterns of *AOX1d*, which peaked in various experimental settings modelling leaf senescence. Since Cd exposure is suggested to induce or accelerate senescence (Srivastava and Jaiswal 1989, Sandalio *et al*. 2001), this is clearly exemplified in our dataset by an increased *AOX1d* expression. In antimycin A-challenged *A. thaliana* seedlings lacking the dominant AOX1a isoform, *AOX1d* expression was induced to a higher extent as compared to the wildtype. Nonetheless, this isoform was unable to fully compensate for the loss of AOX1a, as photosynthesis was still inhibited during antimycin A treatment (Strodtkötter *et al*. 2009). Therefore, the biological significance of *AOX1d* induction is questioned by these authors. However, the substantial induction of *AOX1d* expression observed in our experimental setup does suggest a significant role for this enzyme – next to the major AOX1a isoform – which might not directly be related to optimising photosynthesis. Studying its expression in *Arabidopsis* seedlings without functional AOX1a could reveal more about the potential role of this highly induced gene during Cd exposure, which might be different from its role during antimycin A treatment.

Elhafez *et al*. (2006) demonstrated a coordinated upregulation of *NDA2*, *NDB2* and *AOX1a* under various treatments in *Arabidopsis*, while the expression of *NDB1* and *NDC1* was downregulated under the same conditions. Similarly, we observed a decreasing response for *NDB1* and *NDC1* transcription in both roots and leaves of Cd-exposed seedlings (Supplementary Table 4.4B). In addition, we demonstrated co-expression of specific *ND* and *AOX* isoforms in both roots (Figure 4.4A) and leaves (Figure 4.4B) of Cd-exposed *Arabidopsis* seedlings. To our knowledge, this is the first time that co-expression of *ND* and *AOX* is reported during Cd stress. Previously, Clifton *et al*. (2005) reported co-expression of *NDB2* and *AOX1a* in *Arabidopsis* suspension cells treated with various abiotic stressors over a 24 h time course. This could partly be explained by co-regulation of both genes, as common transcriptional elements with similar organisation occur in the sequences upstream of their coding region (Clifton *et al*. 2005). Ho *et al*. (2008) identified 10 cis-acting regulatory elements (CAREs) in the *Arabidopsis AOX1a* promoter involved in the response to H_2O_2 and/or rotenone treatment, some of which were also functional in the promoters of *NDB2* and *UPOX*. Interestingly, we found a clustering of *AOX1a*, *NDA2* and

NDB2 with *UPOX* in the roots (Figure 4.4A), which suggests a common regulatory pathway controlling the expression of these genes under conditions of Cd exposure in *Arabidopsis*.

Under control conditions, the external ND isoforms *NDB1* and *NDB2* oxidising cytosolic NAD(P)H made up the main part of the *ND* gene family in roots (Figure 4.6C). In the leaves however, *NDA1* and *NDC1* prevailed (Figure 4.6D). This could be linked to the suggested role of both enzymes oxidising matrix NAD(P)H in photorespiration (Bauwe *et al*. 2010). However, a shift toward the external isoforms – and mainly *NDB2* – occurred in Cd-exposed leaves (Figure 4.6D), similarly to the response in roots (Figure 4.6C). As the external oxidation of NADH by *NDB2* is stimulated by Ca^{2+} (Rasmusson *et al*. 2008), the observed increase in external *ND* transcript abundance went hand in hand with a higher Ca content in the roots of plants exposed to 10 µM Cd (Figure 4.2D). External NDs oxidise cytosolic NAD(P)H, which is continuously regenerated by $NAD(P)^+$-reducing enzymes such as isocitrate dehydrogenase (ICDH) and malic enzyme (ME). The capacities of both enzymes were enhanced by Cd in leaves of bean (Van Assche *et al*. 1988, Vangronsveld and Clijsters 1994), maize (Lagriffoul *et al*. 1998) and pepper plants (Léon *et al*. 2002). In addition, Semane *et al*. (2007) showed significant increases in ICDH and ME activities in leaves of *Arabidopsis* seedlings exposed to 10 µM Cd during 7 days. These increases could also reflect the increased demand for cytosolic NAD(P)H to be consumed by external NDs in the alternative ETC during Cd stress.

The abridged ETC can function during the acute response (*i.e.* 24 h) to Cd stress, when increased transcript levels (Table 4.1) were supported by augmented AOX protein contents in roots (at 2 h, Figure 4.5A) and leaves (at 24 h, Figure 4.5B). At later time points however, AOX protein contents decreased as opposed to a sustained elevated transcription in Cd-exposed roots and leaves up to 72 h (Table 4.1). This apparent mismatch could be explained by an increased protein turnover under stress conditions. Similar results were reported during salt stress in *Arabidopsis* (Smith *et al*. 2009). The authors observed a peak in AOX protein after 24 h of salt treatment, which diminished after 72 h. However, *AOX1a* transcripts remained high (Smith *et al*. 2009). The AOX protein is a covalently linked dimer of which both subunits are reversibly linked by a disulfide bond (McDonald 2008). As the protein is more active in the

reduced state, it could be inactivated via oxidation after 48 and 72 h Cd exposure. Therefore, the specific influence of Cd on the mitochondrial redox state should be further investigated.

4.4.4. Concluding remarks

Taken together, our results demonstrate a Cd-induced oxidative challenge at the mitochondrial level and assign a primary role to the alternative respiratory pathway in modulating this challenge, with potential co-regulation of *ND* and *AOX*. Future experiments should reveal if and how these enzymes cooperate in the response and potential acclimation of *Arabidopsis* seedlings to environmentally realistic Cd exposure. In addition, a role for the mitochondrial redox state and reducing power during Cd-induced signalling is evident and deserves further investigation. As both knockout and overexpressor lines for the dominant isoform AOX1a are available, a comparison of their responses to environmentally realistic Cd exposure can shed more light into this potential target and modulator of metal stress in plants.

REFERENCES

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*, **64**, 5245–5250.

Bartoli, C.G., Gomez, F., Gergoff, G., Guiamét, J.J. and Puntarulo, S. (2005) Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions. *J Exp Bot*, **56**, 1269–1276.

Bauwe, H., Hagemann, M. and Fernie, A.R. (2010) Photorespiration: players, partners and origin. *Trends Plant Sci*, **15**, 330–336.

Bi, Y.H., Chen, W.L., Zhang, W.N., Zhou, Q., Yun L.J. and Xing, D. (2009) Production of reactive oxygen species, impairment of photosynthetic function and dynamic changes in mitochondria are early events in cadmium-induced cell death in *Arabidopsis thaliana*. *Biol Cell*, **101**, 629–643.

Borecký, J., Nogueira, F.T.S., de Oliveira, K.A.P., Maia, I.G., Vercesi, A.E. and Arruda, P. (2006) The plant energy-dissipating mitochondrial systems: depicting the genomic structure and the expression profiles of the gene families of uncoupling protein and alternative oxidase in monocots and dicots. *J Exp Bot*, **57**, 849–864.

Castro-Guerrero, N.A., Rodríguez-Zavala, J.S., Marín-Hernández, A., Rodríguez-Enríquez, S. and Moreno-Sánchez, R. (2008) Enhanced alternative oxidase and antioxidant enzymes under Cd^{2+} stress in *Euglena*. *J Bioenerg Biomembr*, **40**, 227–235.

Chen, Z.H. and Niki, E. (2011) Two faces of lipid peroxidation products: the 'Yin and Yang' principles of oxidative stress. *J Exp Integr Med*, **1**, 215–219.

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A. and Whelan, J. (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol Biol*, **58**, 193–212.

Clifton, R., Millar, A.H. and Whelan, J. (2006) Alternative oxidases in Arabidopsis: A comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses. *Biochim Biophys Acta*, **1757**, 730–741.

Collin, V.C., Eymery, F., Genty, B., Rey, P. and Havaux, M. (2008) Vitamin E is essential for the tolerance of *Arabidopsis thaliana* to metal-induced oxidative stress. *Plant Cell Environ*, **31**, 244–257.

Cuypers, A., Vangronsveld, J. and Clijsters, H. (2002) Peroxidases in roots and primary leaves of *Phaseolus vulgaris.* Copper and zinc phytotoxicity: a comparison. *J Plant Physiol*, **159**, 869–876.

Cuypers, A., Smeets, K. and Vangronsveld, J. (2009) Heavy metal stress in plants, in: Hirt, H. (Ed.), Plant stress biology. From genomics to systems biology. Wiley-VCH Verlagsgesellschaft GmbH & Co. KGaA., Weinheim, pp. 161–178.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J. and Smeets, K. (2010) Cadmium stress: an oxidative challenge. *Biometals*, **23**, 927–940.

Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J. and Vangronsveld, J. (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J Plant Physiol*, **168**, 309–316.

Cuypers, A., Keunen, E., Bohler, S., Jozefczak, M., Opdenakker, K., Gielen, H., Vercampt, H., Bielen, A., Schellingen, K., Vangronsveld, J. and Remans, T. (2012) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling, in: Gupta, D.K., Sandalio, L.M. (Eds.), Metal toxicity in plants: Perception, signaling and remediation. Springer-Verlag GmbH, Berlin, Heidelberg, pp. 65–90.

Cvetkovska, M. and Vanlerberghe, G.C. (2012) Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol*, **195**, 32–39.

DalCorso, G., Farinati, S., Maistri, S. and Furini, A. (2008) How plants cope with cadmium: Staking all on metabolism and gene expression. *J Integr Plant Biol*, **50**, 1268–1280.

Duan, Y.P., Yuan, S., Tu, S.H., Feng, W.Q., Xu, F., Zhang, Z.W., Chen, Y.E., Wang, X., Shang, J. and Lin, H.H. (2010) Effects of cadmium stress on alternative oxidase and photosystem II in three wheat cultivars. *Z Naturforsch C*, **65**, 87–94.

Elhafez, D., Murcha, M.W., Clifton, R., Soole, K.L., Day, D.A. and Whelan, J. (2006) Characterization of mitochondrial alternative NAD(P)H dehydrogenases in Arabidopsis: Intraorganelle location and expression. *Plant Cell Physiol*, **47**, 43–54.

Elthon, T.E., Nickels, R.L. and McIntosh, L. (1989) Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. *Plant Physiol*, **89**, 1311–1317.

Finkemeier, I., Goodman, M., Lamkemeyer, P., Kandlbinder, A., Sweetlove, L.J. and Dietz, K.J. (2005) The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of *Arabidopsis thaliana* under stress. *J Biol Chem*, **280**, 12168–12180.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R. and Van Breusegem, F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiol*, **141**, 436–445.

Gallego, S.M., Pena, L.B., Barcia, R.A., Azpilicueta, C.E., Iannone, M.F., Rosales, E.P., Zawoznik, M.S., Groppa, M.D. and Benavides, M.P. (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environ Exp Bot*, **83**, 33–46.

Garmash, E.V. and Golovko, T.K. (2009) Effect of cadmium on growth and respiration of barley plants grown under two temperature regimes. *Russ J Plant Physiol*, **56**, 343–347.

Heyno, E., Klose, C. and Krieger-Liszkay, A. (2008) Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. *New Phytol*, **179**, 687–699.

Ho, L.H.M, Giraud, E., Uggalla, V., Lister, R., Clifton, R., Glen, A., Thirkettle-Watts, D., Van Aken, O. and Whelan, J. (2008) Identification of regulatory pathways controlling gene expression of stress-responsive mitochondrial proteins in Arabidopsis. *Plant Physiol*, **147**, 1858–1873.

Keunen, E., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2011a) Metal-induced oxidative stress and plant mitochondria. *Int J Mol Sci*, **12**, 6894–6918.

Keunen, E., Truyens, S., Bruckers, L., Remans, T., Vangronsveld, J. and Cuypers, A. (2011b) Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged? *Plant Physiol Biochem*, **49**, 1084–1091.

Krznaric, E., Verbruggen, N., Wevers, J.H.L., Carleer, R., Vangronsveld, J. and Colpaert, J.V. (2009) Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd. *Environ Pollut*, **157**, 1581–1588.

Lagriffoul, A., Mocquot, B., Mench, M. and Vangronsveld, J. (1998) Cadmium toxicity effects on growth, mineral and chlorophyll contents, and activities of stress related enzymes in young maize plants (*Zea mays* L.). *Plant Soil*, **200**, 241–250.

Léon, A.M., Palma, J.M., Corpas, F.J., Gómez, M., Romero-Puertas, M.C., Chatterjee, D., Mateos, R.M., del Río, L.A. and Sandalio, L.M. (2002) Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. *Plant Physiol Biochem*, **40**, 813–820.

Martínez-García, J.F., Monte, E. and Quail, P.H. (1999) A simple, rapid and quantitative method for preparing *Arabidopsis* protein extracts for immunoblot analysis. *Plant J*, **20**, 251–257.

Maxwell, D.P., Wang, Y. and McIntosh, L. (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc Natl Acad Sci USA*, **96**, 8271–8276.

McDonald, A.E. (2008) Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. *Funct Plant Biol*, **35**, 535–552.

Morgan, M.J., Lehmann, M., Schwarzländer, M., Baxter, C.J., Sienkiewicz-Porzucek, A., Williams, T.C.R., Schauer, N., Fernie, A.R., Fricker, M.D., Ratcliffe, R.G., Sweetlove, L.J. and Finkemeier, I. (2008) Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. *Plant Physiol*, **147**, 101–114.

Navrot, N., Rouhier, N., Gelhaye, E. and Jacquot, J.P. (2007) Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiol Plant*, **129**, 185–195.

Nawrot, T., Plusquin, M., Hogervorst, J., Roels, H.A., Celis, H., Thijs, L., Vangronsveld, J., Van Hecke, E. and Staessen, J.A. (2006) Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *Lancet Oncol*, **7**, 119–126.

Noctor, G., De Paepe, R. and Foyer, C.H. (2007) Mitochondrial redox biology and homeostasis in plants. *Trends Plant Sci*, **12**, 125–134.

Nogueira, F.T.S., Sassaki, F.T. and Maia, I.G. (2011) *Arabidopsis thaliana* Uncoupling Proteins (AtUCPs): insights into gene expression during development and stress response and epigenetic regulation. *J Bioenerg Biomembr*, **43**, 71–79.

Palmieri, L., Picault, N., Arrigoni, R., Besin, E., Palmieri, F. and Hodges, M. (2008) Molecular identification of three *Arabidopsis thaliana* mitochondrial dicarboxylate carrier isoforms: organ distribution, bacterial expression, reconstitution into liposomes and functional characterization. *Biochem J*, **410**, 621–629.

Pastore, D., Trono, D., Laus, M.N., Di Fonzo, N. and Flagella, Z. (2007) Possible plant mitochondria involvement in cell adaptation to drought stress. A case study: durum wheat mitochondria. *J Exp Bot*, **58**, 195–210.

Polidoros, A.N., Mylona, P.V. and Arnholdt-Schmitt, B. (2009) *Aox* gene structure, transcript variation and expression in plants. *Physiol Plant*, **137**, 342–353.

Rasmusson, A.G., Geisler, D.A. and Møller I.M. (2008) The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. *Mitochondrion*, **8**, 47–60.

Reichheld, J.P., Meyer, E., Khafif, M., Bonnard, G. and Meyer, Y. (2005) AtNTRB is the major mitochondrial thioredoxin reductase in *Arabidopsis thaliana*. *FEBS Lett*, **579**, 337–342.

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J. and Cuypers, A. (2008) Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta*, **227**, 1343–1349.

Sandalio, L.M., Dalurzo, H.C., Gómez, M., Romero-Puertas, M.C. and del Río, L.A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J Exp Bot*, **52**, 2115–2126.

Schwarzländer, M., Fricker, M.D. and Sweetlove, L.J. (2009) Monitoring the *in vivo* redox state of plant mitochondria: Effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. *Biochim Biophys Acta*, **1787**, 468–475.

Semane, B., Cuypers, A., Smeets, K., Van Belleghem, F., Horemans, N., Schat, H. and Vangronsveld, J. (2007) Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. *Physiol Plant*, **129**, 519–528.

Sharma, S.S. and Dietz, K.J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci*, **14**, 43–50.

Smeets, K., Ruytinx, J., Van Belleghem, F., Semane, B., Lin, D., Vangronsveld, J. and Cuypers, A. (2008a) Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. *Plant Physiol Biochem*, **46**, 212–218.

Smeets, K., Ruytinx, J., Semane, B., Van Belleghem, F., Remans, T., Van Sanden, S., Vangronsveld, J. and Cuypers, A. (2008b) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environ Exp Bot*, **63**, 1–8.

Smith, C.A., Melino, V.J., Sweetman, C. and Soole, K.S. (2009) Manipulation of alternative oxidase can influence salt tolerance in *Arabidopsis thaliana*. *Physiol Plant*, **137**, 459–472.

Srivastava, A. and Jaiswal, V.S. (1989) Biochemical changes in duck weed after cadmium treatment. Enhancement in senescence. *Water, Air, Soil Pollut*, **50**, 163–170.

Strodtkötter, I., Padmasree, K., Dinakar, C., Speth, B., Niazi, P.S., Wojtera, J., Voss, I., Do, P.T., Nunes-Nesi, A., Fernie, A.R., Linke, V., Raghavendra, A.S. and Scheibe, R. (2009) Induction of the AOX1D isoform of alternative oxidase in *A. thaliana* T-DNA insertion lines lacking isoform AOX1A is insufficient to optimize photosynthesis when treated with antimycin A. *Mol Plant*, **2**, 284–297.

Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J. and Millar, A.H. (2002) The impact of oxidative stress on *Arabidopsis* mitochondria. *Plant J*, **32**, 891–904.

Van Aken, O., Zhang, B., Carrie, C., Uggalla, V., Paynter, E., Giraud, E. and Whelan, J. (2009) Defining the mitochondrial stress response in *Arabidopsis thaliana*. *Mol Plant*, **2**, 1310–1324.

Van Assche, F., Cardinaels, C. and Clijsters, H. (1988) Induction of enzyme capacity in plants as a result of heavy metal toxicity: Dose-response relations in *Phaseolus vulgaris* L., treated with zinc and cadmium. *Environ Pollut*, **52**, 103–115.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, **3**, research0034.1–034.11.

Vangronsveld, J. and Clijsters, H. (1994) Toxic effects of metals, in: Farago, M.E. (Ed.), Plants and the chemical elements. VCH Verlagsgesellschaft GmbH, Weinheim, pp. 149–177.

Vanlerberghe, G.C., Cvetkovska, M. and Wang, J. (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? *Physiol Plant*, **137**, 392–406.

Vercesi, A.E., Borecký, J., de Godoy Maia, I., Arruda, P., Cuccovia, I.M. and Chaimovich, H. (2006) Plant uncoupling mitochondrial proteins. *Annu Rev Plant Biol*, **57**, 383–404.

Wang, X., Zhang, Z.W., Tu, S.H., Feng, W.Q., Xu, F., Zhu, F., Zhang, D.W., Du, J.B., Yuan, S. and Lin, H.H. (2013) Comparative study of four rice cultivars with different levels of cadmium tolerance. *Biologia*, **68**, 74–81.

Watanabe, C.K., Hachiya, T., Takahara, K., Kawai-Yamada, M., Uchimiya, H., Uesono, Y., Terashima, I. and Noguchi, K. (2010) Effects of AOX1a deficiency on plant growth, gene expression of respiratory components and metabolic profile under low-nitrogen stress in *Arabidopsis thaliana*. *Plant Cell Physiol*, **51**, 810–822.

Yoshida, K. and Noguchi, K. (2009) Differential gene expression profiles of the mitochondrial respiratory components in illuminated Arabidopsis leaves. *Plant Cell Physiol*, **50**, 1449–1462.

Yoshida, K., Watanabe, C.K., Hachiya, T., Tholen, D., Shibata, M., Terashima, I. and Noguchi, K. (2011) Distinct responses of the mitochondrial respiratory chain to long- and short-term high-light environments in *Arabidopsis thaliana*. *Plant Cell Environ*, **34**, 618–628.

SUPPLEMENTARY INFORMATION

Supplementary Table 4.1. *The sequences of the forward and reverse primers used to determine gene expression levels via quantitative real-time PCR. UBC, ubiquitin-conjugating enzyme; TIP, tonoplast intrinsic protein; PPR, pentatricopeptide repeat; ACT, actin; UPOX, upregulated by oxidative stress; MSD, manganese superoxide dismutase; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GDXN, glutaredoxin; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; NTR, NADPH-dependent thioredoxin reductase; Prx, peroxiredoxin; Trx, thioredoxin; CI76, 76 kDa subunit of complex I; SHD2-1, succinate dehydrogenase 2-1 in complex II; COX5b, 5b subunit of cytochrome c oxidase (complex IV); COX6b, 6b subunit of cytochrome c oxidase (complex IV); AOX, alternative oxidase; ND, alternative NAD(P)H dehydrogenase; UCP, uncoupling protein.*

AGI	***Annotation***	***Forward primer (5'-3')***	***Reverse primer (5'-3')***
Reference genes			
AT5G25760	*UBC*	*CTGCGACTCAGGGAATCTTCTAA*	*TTGTGCCATTGAATTGAACCC*
AT2G28390	*SAND family*	*AACTCTATGCAGCATTTGATCCACT*	*TGATTGCATATCTTTATCGCCATC*
AT4G34270	*TIP41-like*	*GTGAAAACTGTTGGAGAGAAGCAA*	*TCAACTGGATACCCTTTCGCA*
AT5G55840	*PPR gene*	*AAGACAGTGAAGGTGCAACCTTACT*	*AGTTTTTGAGTTGTATTTGTCAGAGAAAG*
AT3G18780	*ACT2*	*CTTGCACCAAGCAGCATGAA*	*CCGATCCAGACACTGTACTTCCTT*
Gene encoding a mitochondrial marker protein for oxidative stress			
AT2G21640	*UPOX*	*GACTTGTTTCAAAAACACCATGGAC*	*CACTTCCTTAGCCTCAATTTGCTTC*
Genes encoding mitochondrial antioxidative enzymes			
AT3G10920	*MSD1*	*ATGTTTGGGAGCACGCCTAC*	*AACCTCGCTTGCATATTTCCA*
AT4G08390	*APX*	*GGACACCAGAGTGGCTGAAGTT*	*GCATCAGTGGGTAGGACAAGGAG*
AT1G19570	*DHAR*	*GATGGCTCTGGAAATCTGTGTGA*	*CGAGTGTGAGAAGAGCCCGT*
AT3G15660	*GDXN*	*AGACCAAGAGTTGAAAAACGCTGT*	*TGAGCCGCCAATGAACTCTC*
AT3G54660	*GR*	*TAGGGTTGGAGAATGTTGGCG*	*GCCCAGATGGATGGAACAGAT*
AT1G63940	*MDHAR*	*TAGTAGAAAGCGGATCGCCTGA*	*GCGCTTGCGAGTTTAGCCTT*
AT2G17420	*NTRA*	*TTTGCTGCTGGAGATGTTCAAG*	*AATGCTCTGCATCCAATGCC*
AT4G35460	*NTRB*	*GAGTTTTCGCTGCGGGTGA*	*TCTGCATCCAAAGCTGCCAT*
AT3G06050	*Prx*	*GCTATCAATGGTTGGGCAGAGA*	*GCCCCAAGCTTTTGTGAAATT*
AT2G35010	*Trx*	*AGGGCGGGATTTCGAACAC*	*ACCTCCCCTTTCTTCGAGCC*
Genes encoding mitochondrial phosphorylating respiratory enzymes			
AT5G37510	*CI76*	*AGCCTGTAGCTTCATGTGCCA*	*ATCACTCCCTCCCTCGCCTT*
AT3G27380	*SDH2-1*	*CCAGCTGCTTTGCTACACGC*	*TGAACTCGTCGTCAATAGCCTCA*
AT3G15640	*COX5b*	*AGTCCTTTGAATGCCCGGTT*	*TCGTCTTCGTCACCGTGACC*
AT1G22450	*COX6b*	*ACTTTCAATGGCGGATGCTG*	*TGCACTTGTGTCTTGCTTCACTTC*
Genes encoding alternative oxidases (AOX)			
AT3G22370	*AOX1a*	*CTCTTCGTTGGCCTACCGATT*	*AACCATTCCAGGTACTGCTGCTAC*
AT3G22360	*AOX1b*	*GGACAAACTAGCTTATTGGACCGTG*	*TCATTGCTCTGCATCCGTACC*
AT3G27620	*AOX1c*	*GGTGGTTCGTGCTGATGAGG*	*CTTCTTTCAGCTCATGACCTTGG*
AT1G32350	*AOX1d*	*ACCGTTCAAACTCTGAAAATACCG*	*GCAGCCACCGTCTCTAGCAA*

Supplementary Table 4.1. *Continued.*

AGI	Annotation	Forward primer (5'-3')	Reverse primer (5'-3')
Genes encoding alternative NAD(P)H dehydrogenases (ND)			
AT1G07180	NDA1	GCAAGGAAGGGAAAGGAATATCA	TTTCTCCAGCTGACGACTCGA
AT2G29990	NDA2	TAGTCGACCTTCGCGAGAGC	TCGAGTCAGGTACGCCGATC
AT4G28220	NDB1	ATATGCAGTTGGCGATTGTGC	TGAGTTGTCCGCATCTGCAG
AT4G05020	NDB2	GTCCAGAAGGTCCCATTAGGATG	CCCAGTGGTGCAAATTGTCC
AT4G21490	NDB3	GCTTCTACCAGCGACAGGACA	CAGGATTCTTCTCGCATACTTCCA
AT2G20800	NDB4	CAGGAATTGGTTCACGTCCTGT	CTAAGCCATTCGTCGGTAGCC
AT5G08740	NDC1	CGAACGTTTTGTGTTCAAGCC	AAGCAAATCCGAGAAACGGG
Genes encoding uncoupling proteins (UCP)			
AT3G54110	UCP1	AGGTGCGCCAAGACGGTATT	TCCAAGACCAGTCCAAAGAGCTC
AT5G58970	UCP2	CGTTCCTTGAAACCTTCATTTGC	TGAAGTCTAACTTTGGCTGTGTCCA
AT1G14140	UCP3	ACAAAGGCTCTCGTCGGAGG	CATCTGCTTGCATTCTCACTTTGA
AT4G24570	UCP4	TGTGCGGTGAAGACGGTTAAA	CAACAGTGAAAGGACCTTGCCT
AT2G22500	UCP5	GACCCACCCGCTTGATCTAATC	AAAAGCAAGAGCTGGTCGGAG

Supplementary Table 4.2. *Exposure to Cd has a minimal effect on the expression levels of genes encoding antioxidative defence enzymes that are specifically expressed in mitochondria in Arabidopsis roots and leaves. Analyses were performed using quantitative real-time PCR. Transcript levels were measured in roots and leaves of Arabidopsis thaliana plants exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 4 biological replicates relative to the control (0 µM) at each time point set at 1.00. Significance levels relative to the control: $p < 0.05$; $p < 0.01$; $p < 0.05$; $p < 0.01$ for induction and inhibition respectively. APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GDXN, glutaredoxin; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; NTR, NADPH-dependent thioredoxin reductase; Prx, peroxiredoxin; Trx, thioredoxin.*

Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
ROOTS						
APX	0	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.16	1.00 ± 0.01	1.00 ± 0.04
	5		0.77 ± 0.20	0.88 ± 0.01	0.61 ± 0.12	0.48 ± 0.10
	10		0.59 ± 0.16	0.59 ± 0.06	0.55 ± 0.03	0.49 ± 0.06
DHAR	0	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.10	1.00 ± 0.19	1.00 ± 0.04
	5		0.80 ± 0.15	1.20 ± 0.23	0.95 ± 0.21	1.08 ± 0.31
	10		1.04 ± 0.22	0.62 ± 0.05	0.62 ± 0.09	0.66 ± 0.12
GDXN	0	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.14	1.00 ± 0.03
	5		1.07 ± 0.07	1.09 ± 0.14	1.21 ± 0.16	1.00 ± 0.01
	10		1.30 ± 0.19	1.33 ± 0.12	1.11 ± 0.02	1.19 ± 0.09
GR	0	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.21	1.00 ± 0.03	1.00 ± 0.05
	5		0.65 ± 0.17	0.91 ± 0.17	0.58 ± 0.30	0.45 ± 0.17
	10		0.58 ± 0.25	0.76 ± 0.13	0.48 ± 0.08	0.31 ± 0.01
MDHAR	0	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.02	1.00 ± 0.19	1.00 ± 0.08
	5		0.69 ± 0.00	0.68 ± 0.12	0.53 ± 0.14	0.47 ± 0.11
	10		0.72 ± 0.11	0.54 ± 0.10	0.46 ± 0.08	0.33 ± 0.05
NTRA	0	1.00 ± 0.04	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.06
	5		1.17 ± 0.13	0.93 ± 0.09	1.23 ± 0.27	1.22 ± 0.21
	10		0.95 ± 0.16	1.51 ± 0.19	1.83 ± 0.10	1.74 ± 0.17
NTRB	0	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03
	5		0.98 ± 0.09	0.86 ± 0.05	0.89 ± 0.09	0.78 ± 0.15
	10		0.74 ± 0.16	0.90 ± 0.08	0.77 ± 0.06	0.94 ± 0.07
Prx	0	1.00 ± 0.09	1.00 ± 0.04	1.00 ± 0.15	1.00 ± 0.08	1.00 ± 0.04
	5		1.13 ± 0.21	1.08 ± 0.02	0.96 ± 0.19	0.60 ± 0.11
	10		0.86 ± 0.16	0.98 ± 0.07	0.72 ± 0.06	0.48 ± 0.05
Trx	0	1.00 ± 0.03	1.00 ± 0.10	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.02
	5		1.04 ± 0.07	0.99 ± 0.11	1.05 ± 0.10	1.08 ± 0.08
	10		1.25 ± 0.15	1.21 ± 0.10	1.25 ± 0.08	1.29 ± 0.07

Supplementary Table 4.2. *Continued.*

Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
LEAVES						
APX	0	1.00 ± 0.07	1.00 ± 0.16	1.00 ± 0.13	1.00 ± 0.09	1.00 ± 0.07
	5		1.13 ± 0.21	0.62 ± 0.09	1.19 ± 0.09	0.85 ± 0.08
	10		1.25 ± 0.06	0.69 ± 0.09	1.01 ± 0.10	0.91 ± 0.11
DHAR	0	1.00 ± 0.12	1.00 ± 0.28	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.16
	5		0.55 ± 0.10	1.35 ± 0.20	1.37 ± 0.08	1.54 ± 0.03
	10		0.75 ± 0.23	2.42 ± 0.22	2.62 ± 0.07	3.81 ± 0.76
GDXN	0	1.00 ± 0.09	1.00 ± 0.19	1.00 ± 0.24	1.00 ± 0.18	1.00 ± 0.12
	5		1.21 ± 0.20	0.97 ± 0.09	1.48 ± 0.08	1.18 ± 0.11
	10		1.33 ± 0.24	1.21 ± 0.19	1.68 ± 0.19	1.34 ± 0.19
GR	0	1.00 ± 0.13	1.00 ± 0.22	1.00 ± 0.15	1.00 ± 0.18	1.00 ± 0.16
	5		1.42 ± 0.23	0.79 ± 0.02	0.98 ± 0.02	1.13 ± 0.10
	10		1.28 ± 0.22	0.45 ± 0.08	0.69 ± 0.08	1.16 ± 0.23
MDHAR	0	1.00 ± 0.06	1.00 ± 0.19	1.00 ± 0.11	1.00 ± 0.09	1.00 ± 0.03
	5		1.34 ± 0.13	0.49 ± 0.06	0.95 ± 0.01	0.81 ± 0.04
	10		1.46 ± 0.21	0.39 ± 0.05	0.75 ± 0.07	0.73 ± 0.06
NTRA	0	1.00 ± 0.08	1.00 ± 0.17	1.00 ± 0.23	1.00 ± 0.18	1.00 ± 0.08
	5		1.10 ± 0.14	1.83 ± 0.30	1.56 ± 0.08	1.23 ± 0.16
	10		1.23 ± 0.16	3.55 ± 0.89	2.47 ± 0.46	1.59 ± 0.26
NTRB	0	1.00 ± 0.03	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.02	1.00 ± 0.09
	5		1.23 ± 0.11	0.68 ± 0.03	0.88 ± 0.01	0.76 ± 0.05
	10		1.19 ± 0.07	1.12 ± 0.23	0.96 ± 0.09	0.64 ± 0.06
Prx	0	1.00 ± 0.09	1.00 ± 0.15	1.00 ± 0.08	1.00 ± 0.14	1.00 ± 0.11
	5		1.06 ± 0.11	1.39 ± 0.16	2.19 ± 0.02	1.34 ± 0.13
	10		1.04 ± 0.03	1.37 ± 0.15	1.94 ± 0.13	1.73 ± 0.20
Trx	0	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.13	1.00 ± 0.06	1.00 ± 0.07
	5		1.05 ± 0.08	1.04 ± 0.11	1.29 ± 0.03	0.97 ± 0.09
	10		1.10 ± 0.14	1.17 ± 0.30	1.34 ± 0.02	1.04 ± 0.13

Supplementary Table 4.3. *Exposure to Cd has a minimal effect on the expression levels of genes encoding mitochondrial phosphorylating respiratory enzymes in Arabidopsis roots and leaves. Analyses were performed using quantitative real-time PCR. Transcript levels were measured in roots and leaves of Arabidopsis thaliana plants exposed to 5 or 10 μM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 4 biological replicates relative to the control (0 μM) at each time point set at 1.00. Significance levels relative to the control: $p < 0.05$; $p < 0.01$; $p < 0.05$; $p < 0.01$ for induction and inhibition respectively. CI76, 76 kDa subunit of complex I; SHD2-1, succinate dehydrogenase 2-1 in complex II; COX5b, 5b subunit of cytochrome c oxidase (complex IV); COX6b, 6b subunit of cytochrome c oxidase (complex IV).*

Gene	$CdSO_4$ (μM)	0 h	2 h	24 h	48 h	72 h
ROOTS						
CI76	0	1.00 ± 0.05	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.01	1.00 ± 0.05
	5		0.92 ± 0.11	0.75 ± 0.19	0.71 ± 0.17	0.56 ± 0.17
	10		0.81 ± 0.25	0.66 ± 0.05	0.59 ± 0.05	0.44 ± 0.08
SHD2-1	0	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.08	1.00 ± 0.01
	5		0.92 ± 0.03	1.10 ± 0.09	1.07 ± 0.19	0.89 ± 0.08
	10		0.84 ± 0.02	1.21 ± 0.11	1.01 ± 0.07	1.19 ± 0.06
COX5b	0	1.00 ± 0.03	1.00 ± 0.11	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.05
	5		1.25 ± 0.12	0.93 ± 0.16	0.92 ± 0.16	0.79 ± 0.08
	10		1.21 ± 0.18	1.16 ± 0.09	0.91 ± 0.04	0.65 ± 0.08
COX6b	0	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.19	1.00 ± 0.02	1.00 ± 0.02
	5		0.83 ± 0.22	0.96 ± 0.02	1.00 ± 0.45	0.81 ± 0.31
	10		0.81 ± 0.21	0.56 ± 0.09	0.35 ± 0.05	0.28 ± 0.03
LEAVES						
CI76	0	1.00 ± 0.07	1.00 ± 0.21	1.00 ± 0.05	1.00 ± 0.09	1.00 ± 0.00
	5		1.05 ± 0.09	1.03 ± 0.10	1.51 ± 0.10	1.26 ± 0.05
	10		1.12 ± 0.09	0.92 ± 0.01	1.58 ± 0.09	1.59 ± 0.19
SHD2-1	0	1.00 ± 0.03	1.00 ± 0.13	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.05
	5		1.02 ± 0.00	2.13 ± 0.43	2.69 ± 0.33	1.63 ± 0.29
	10		1.02 ± 0.03	4.42 ± 1.22	5.74 ± 1.29	4.18 ± 0.97
COX5b	0	1.00 ± 0.07	1.00 ± 0.14	1.00 ± 0.04	1.00 ± 0.13	1.00 ± 0.11
	5		1.10 ± 0.12	1.27 ± 0.17	0.96 ± 0.09	0.81 ± 0.03
	10		1.26 ± 0.19	1.39 ± 0.10	0.67 ± 0.03	0.38 ± 0.03
COX6b	0	1.00 ± 0.11	1.00 ± 0.18	1.00 ± 0.12	1.00 ± 0.17	1.00 ± 0.14
	5		1.16 ± 0.10	1.08 ± 0.06	1.21 ± 0.06	1.21 ± 0.08
	10		1.04 ± 0.13	0.97 ± 0.12	0.97 ± 0.08	1.01 ± 0.17

Supplementary Table 4.4. *Exposure to Cd influences the expression levels of genes encoding different components of the mitochondrial alternative respiratory pathway in Arabidopsis roots and leaves. Analyses were performed using quantitative real-time PCR. Transcript levels were measured in roots and leaves of Arabidopsis thaliana plants exposed to 5 or 10 μM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 4 biological replicates relative to the control (0 μM) at each time point set at 1.00. Significance levels relative to the control: $p < 0.05$; $p < 0.01$; $p < 0.05$; $p < 0.01$ for induction and inhibition respectively. (A) The effects of Cd exposure on the expression of AOX1 genes in A. thaliana. (B) The effects of Cd exposure on the expression of alternative ND genes in A. thaliana. (C) The effects of Cd exposure on the expression of UCP genes in A. thaliana. AOX, alternative oxidase; ND, alternative NAD(P)H dehydrogenase; UCP, uncoupling protein.*

Supplementary Table 4.4. Part A.

Gene	$CdSO_4$ (μM)	0 h	2 h	24 h	48 h	72 h
ROOTS						
AOX1a	0	1.00 ± 0.09	1.00 ± 0.14	1.00 ± 0.12	1.00 ± 0.06	1.00 ± 0.05
	5		1.81 ± 0.27	2.75 ± 0.36	2.47 ± 0.52	1.34 ± 0.19
	10		1.71 ± 0.22	5.12 ± 1.12	7.34 ± 0.66	5.11 ± 0.91
AOX1c	0	1.00 ± 0.08	1.00 ± 0.16	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.06
	5		0.83 ± 0.17	0.70 ± 0.10	0.58 ± 0.08	0.49 ± 0.14
	10		0.68 ± 0.07	0.47 ± 0.18	0.61 ± 0.08	0.64 ± 0.24
LEAVES						
AOX1a	0	1.00 ± 0.06	1.00 ± 0.17	1.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.10
	5		0.96 ± 0.11	7.76 ± 0.28	3.37 ± 0.43	2.40 ± 0.52
	10		1.13 ± 0.11	11.85 ± 2.57	7.55 ± 1.43	5.74 ± 1.57
AOX1d	0	1.00 ± 0.81	1.00 ± 0.67	1.00 ± 0.38	1.00 ± 0.66	1.00 ± 0.32
	5		2.54 ± 1.77	2804.08 ± 965.74	83.36 ± 25.27	203.68 ± 94.97
	10		16.92 ± 9.08	5838.49 ± 1247.11	302.70 ± 78.58	693.16 ± 190.44

Supplementary Table 4.4. Part B (roots).

Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
ROOTS						
NDA1	0	1.00 ± 0.14	1.00 ± 0.59	1.00 ± 0.23	1.00 ± 0.07	1.00 ± 0.12
	5		0.51 ± 0.12	1.24 ± 0.08	1.13 ± 0.29	0.82 ± 0.22
	10		0.46 ± 0.12	2.08 ± 0.37	1.39 ± 0.47	1.56 ± 0.37
NDA2	0	1.00 ± 0.05	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.07
	5		0.90 ± 0.09	1.77 ± 0.25	1.42 ± 0.11	1.29 ± 0.19
	10		0.87 ± 0.12	4.47 ± 0.68	4.02 ± 0.38	5.81 ± 0.13
NDB1	0	1.00 ± 0.11	1.00 ± 0.09	1.00 ± 0.17	1.00 ± 0.01	1.00 ± 0.04
	5		1.11 ± 0.22	0.96 ± 0.15	0.67 ± 0.19	0.48 ± 0.13
	10		0.84 ± 0.18	0.49 ± 0.04	0.38 ± 0.05	0.23 ± 0.03
NDB2	0	1.00 ± 0.06	1.00 ± 0.11	1.00 ± 0.24	1.00 ± 0.19	1.00 ± 0.07
	5		1.28 ± 0.12	1.64 ± 0.29	1.64 ± 0.23	0.99 ± 0.25
	10		1.11 ± 0.25	2.29 ± 0.36	3.81 ± 0.12	2.23 ± 0.03
NDB4	0	1.00 ± 0.17	1.00 ± 0.16	1.00 ± 0.30	1.00 ± 0.30	1.00 ± 0.20
	5		1.05 ± 0.23	5.12 ± 0.98	8.84 ± 4.54	2.03 ± 0.41
	10		1.10 ± 0.54	13.64 ± 5.12	7.64 ± 3.45	5.84 ± 1.52
NDC1	0	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.15	1.00 ± 0.02	1.00 ± 0.03
	5		0.86 ± 0.12	0.99 ± 0.02	1.08 ± 0.09	0.77 ± 0.11
	10		0.92 ± 0.15	0.86 ± 0.07	0.81 ± 0.05	0.62 ± 0.03

Supplementary Table 4.4. Part B (leaves).

Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
LEAVES						
NDA1	0	1.00 ± 0.07	1.00 ± 0.23	1.00 ± 0.24	1.00 ± 0.13	1.00 ± 0.14
	5		1.62 ± 0.29	0.45 ± 0.15	0.62 ± 0.10	0.83 ± 0.05
	10		1.62 ± 0.27	0.32 ± 0.06	0.45 ± 0.02	0.43 ± 0.06
NDA2	0	1.00 ± 0.05	1.00 ± 0.38	1.00 ± 0.10	1.00 ± 0.28	1.00 ± 0.03
	5		0.82 ± 0.05	14.10 ± 3.27	5.12 ± 0.09	3.54 ± 0.37
	10		1.10 ± 0.15	23.50 ± 4.37	7.50 ± 0.94	6.95 ± 0.79
NDB1	0	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.04
	5		1.15 ± 0.08	0.88 ± 0.02	1.00 ± 0.08	1.14 ± 0.07
	10		1.13 ± 0.11	0.43 ± 0.02	0.59 ± 0.14	0.70 ± 0.15
NDB2	0	1.00 ± 0.06	1.00 ± 0.36	1.00 ± 0.05	1.00 ± 0.25	1.00 ± 0.01
	5		0.73 ± 0.06	11.07 ± 1.77	3.61 ± 0.08	2.63 ± 0.26
	10		1.06 ± 0.16	17.28 ± 1.32	5.03 ± 0.42	3.86 ± 0.57
NDB3	0	1.00 ± 0.22	1.00 ± 0.23	1.00 ± 0.59	1.00 ± 0.37	1.00 ± 0.32
	5		0.50 ± 0.15	1.18 ± 0.30	2.27 ± 0.68	1.99 ± 0.22
	10		1.13 ± 0.41	1.71 ± 0.89	0.93 ± 0.06	2.56 ± 0.70
NDB4	0	1.00 ± 0.16	1.00 ± 0.35	1.00 ± 0.25	1.00 ± 0.51	1.00 ± 0.20
	5		0.30 ± 0.04	6.77 ± 2.40	15.14 ± 4.00	19.61 ± 3.63
	10		0.81 ± 0.25	10.61 ± 2.82	10.50 ± 2.06	11.22 ± 4.12
NDC1	0	1.00 ± 0.03	1.00 ± 0.25	1.00 ± 0.09	1.00 ± 0.02	1.00 ± 0.07
	5		1.25 ± 0.09	0.71 ± 0.08	0.74 ± 0.03	0.95 ± 0.04
	10		1.24 ± 0.07	0.44 ± 0.03	0.69 ± 0.03	0.74 ± 0.05

Supplementary Table 4.4. Part C (roots).

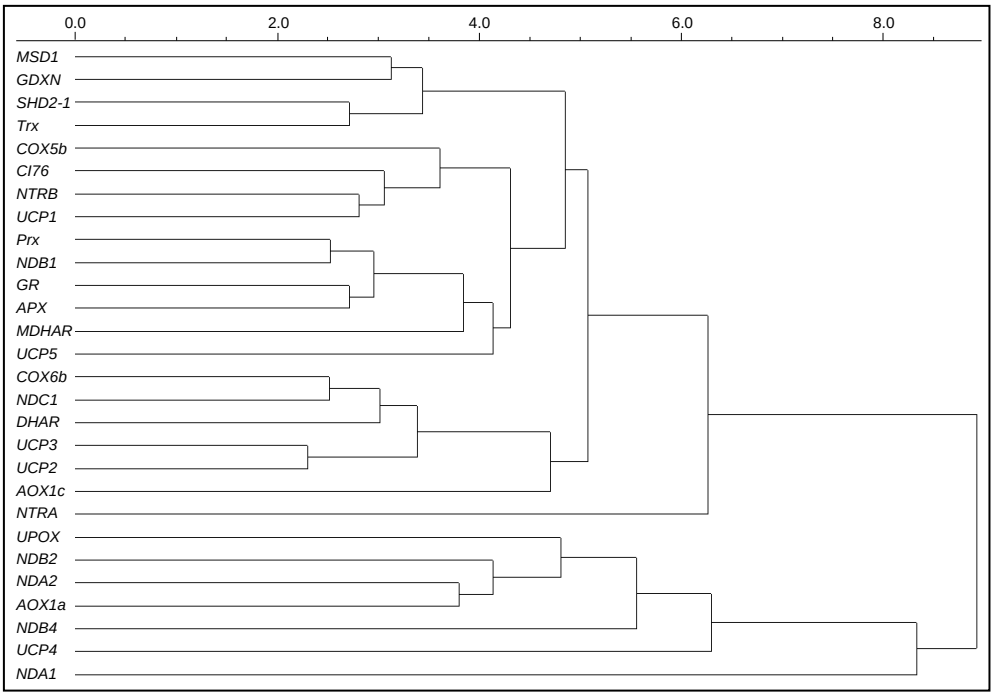
Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
ROOTS						
UCP1	0	1.00 ± 0.08	1.00 ± 0.08	1.00 ± 0.14	1.00 ± 0.03	1.00 ± 0.08
	5		0.80 ± 0.11	1.12 ± 0.08	1.00 ± 0.20	0.61 ± 0.17
	10		0.70 ± 0.22	1.34 ± 0.17	0.86 ± 0.06	0.78 ± 0.05
UCP2	0	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.01	1.00 ± 0.06
	5		0.81 ± 0.14	0.91 ± 0.14	1.02 ± 0.26	0.64 ± 0.12
	10		0.64 ± 0.18	0.43 ± 0.11	0.43 ± 0.07	0.46 ± 0.10
UCP3	0	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.07
	5		0.80 ± 0.11	1.37 ± 0.26	1.16 ± 0.20	0.80 ± 0.09
	10		0.88 ± 0.12	0.84 ± 0.17	0.57 ± 0.07	0.48 ± 0.06
UCP4	0	1.00 ± 0.14	1.00 ± 0.11	1.00 ± 0.21	1.00 ± 0.35	1.00 ± 0.09
	5		1.62 ± 0.25	1.98 ± 0.16	0.50 ± 0.20	1.35 ± 0.41
	10		1.28 ± 0.41	2.29 ± 0.08	1.51 ± 0.46	1.86 ± 0.28
UCP5	0	1.00 ± 0.11	1.00 ± 0.12	1.00 ± 0.23	1.00 ± 0.13	1.00 ± 0.05
	5		1.22 ± 0.22	1.13 ± 0.18	0.80 ± 0.17	0.62 ± 0.16
	10		1.10 ± 0.32	0.85 ± 0.15	0.83 ± 0.16	0.54 ± 0.04

Supplementary Table 4.4. Part C (leaves).

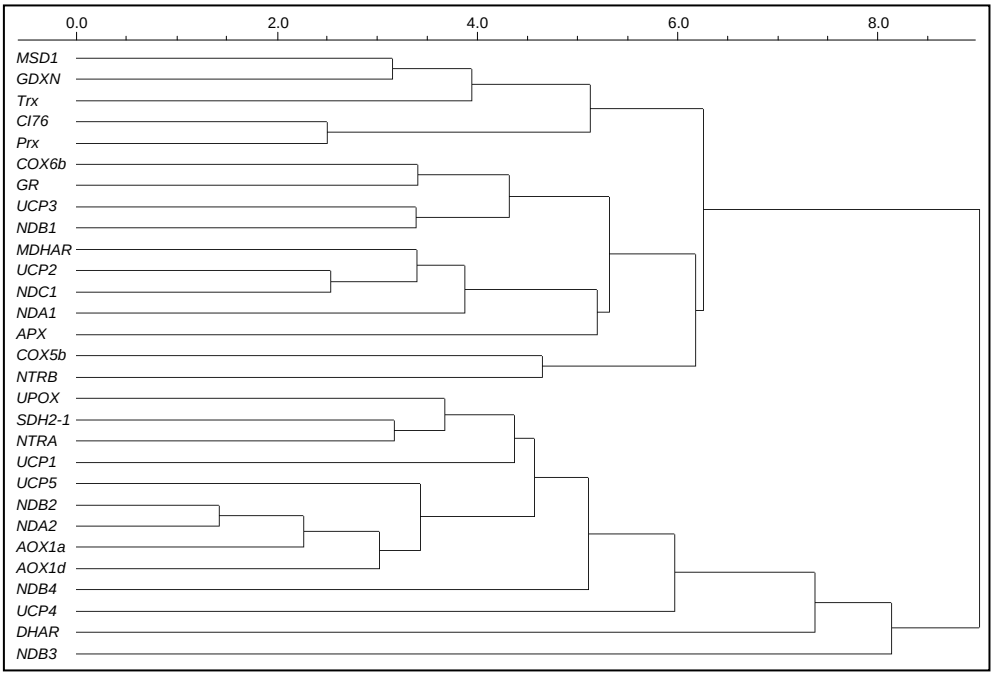
Gene	$CdSO_4$ (µM)	0 h	2 h	24 h	48 h	72 h
LEAVES						
UCP1	0	1.00 ± 0.06	1.00 ± 0.34	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.02
	5		1.34 ± 0.09	3.00 ± 0.51	1.62 ± 0.11	1.34 ± 0.14
	10		1.34 ± 0.07	3.89 ± 0.48	1.95 ± 0.21	1.66 ± 0.09
UCP2	0	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.02
	5		1.23 ± 0.08	0.55 ± 0.06	0.91 ± 0.02	1.09 ± 0.05
	10		1.07 ± 0.08	0.32 ± 0.06	0.73 ± 0.04	0.85 ± 0.12
UCP3	0	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.05
	5		1.09 ± 0.10	0.90 ± 0.08	0.96 ± 0.06	1.08 ± 0.13
	10		1.00 ± 0.10	0.63 ± 0.11	0.71 ± 0.04	0.72 ± 0.10
UCP4	0	1.00 ± 0.17	1.00 ± 0.20	1.00 ± 0.23	1.00 ± 0.42	1.00 ± 0.24
	5		0.84 ± 0.15	5.58 ± 1.51	0.51 ± 0.19	3.31 ± 0.32
	10		0.85 ± 0.17	3.73 ± 0.46	0.89 ± 0.36	1.41 ± 0.25
UCP5	0	1.00 ± 0.11	1.00 ± 0.46	1.00 ± 0.10	1.00 ± 0.17	1.00 ± 0.09
	5		0.62 ± 0.08	10.00 ± 1.75	2.17 ± 0.09	3.69 ± 0.42
	10		1.03 ± 0.24	7.69 ± 0.63	2.60 ± 0.46	3.21 ± 0.17

Supplementary Figure 4.1.

A



B



Supplementary Figure 4.1. *Hierarchical clustering analysis of all measured mitochondrial antioxidative and respiratory genes revealed co-expression of specific components of the mitochondrial alternative respiratory chain in (A) roots and (B) leaves of Arabidopsis thaliana plants exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. The plots shown are based on raw gene expression values and the "Unweighted pairs linkage" algorithm, which defines the distance between groups (treatments) as the average of the distances between all pairs of individuals in all groups. Distances are calculated based on the Euclidian Distance Measure. Clusters obtained with the other algorithms (single and complete linkage and Ward's algorithm) were similar and therefore not shown.*

CHAPTER 5

ALTERNATIVE OXIDASE1a modulates the oxidative challenge during moderate Cd exposure in *Arabidopsis thaliana* leaves

Els Keunen, Kerim Schellingen, Dominique Van Der Straeten, Tony Remans, Jaco Vangronsveld, Ann Cuypers (2014) ALTERNATIVE OXIDASE1a modulates the oxidative challenge during moderate Cd exposure in *Arabidopsis thaliana* leaves. Submitted to Journal of Experimental Botany.

ABSTRACT

This study aims to unravel the functional significance of alternative oxidase 1a (AOX1a) induction in cadmium (Cd)-exposed *Arabidopsis thaliana* leaves by comparing wild-type (WT) plants and *aox1a* knockout mutants. In the absence of AOX1a, differences in stress-responsive transcript and glutathione levels suggest an increased oxidative challenge during moderate (5 µM) and prolonged (72 h) Cd exposure. Nevertheless, *aox1a* knockout leaves showed lower hydrogen peroxide (H_2O_2) accumulation as compared to the WT during both acute (24 h) and prolonged (72 h) exposure to 5 but not 10 µM Cd. Taken together, we propose a working model where AOX1a acts early in the response to Cd and activates or maintains a mitochondrial signalling pathway impacting on cellular antioxidative defence at post-transcriptional level. This fine-tuning pathway potentially involves H_2O_2 as retrograde signal and is suggested to function during moderate (5 µM) Cd exposure while being overwhelmed during more severe (10 µM) Cd stress. Within this framework, ethylene is required – either directly or indirectly via NADPH oxidase isoform C – to fully induce *AOX1* expression. In addition, a reciprocal crosstalk between these components was demonstrated in *A. thaliana* leaves under Cd exposure.

Keywords:

alternative oxidase, alternative respiration, *Arabidopsis thaliana*, cadmium, ethylene, oxidative challenge.

5.1. INTRODUCTION

In the plant mitochondrial electron transport chain (ETC), two terminal oxidases are able to reduce O_2 to H_2O. As opposed to cytochrome c oxidase (complex IV), the alternative oxidase (AOX) does not translocate protons across the inner membrane. As AOX bypasses proton-pumping complexes III and IV, the energy (ATP) yield is reduced (Millar *et al*. 2011). Under normal conditions, AOX is suggested to modulate the production of reactive oxygen species (ROS) (Vanlerberghe *et al*. 2009), although conflicting results appear in literature. Amirsadeghi *et al*. (2006) have reported diminished steady-state cellular ROS levels in tobacco leaves lacking AOX as compared to wild-type (WT) leaves. However, Cvetkovska and Vanlerberghe (2012) showed that a lack of AOX increases superoxide ($O_2^{\bullet -}$) levels in tobacco leaf mitochondria. Nevertheless, these data support the long-standing hypothesis that AOX modulates the production of mitochondrial ROS in plants (Purvis and Shewfelt 1993).

The relationship between ROS and AOX might also imply a function for this enzyme during abiotic stress conditions, supported by the fact that expression of the dominant *AOX1a* isoform in *Arabidopsis thaliana* is highly stress-responsive (Clifton *et al*. 2005, Vanlerberghe *et al*. 2009). Abiotic stress is often characterised by an oxidative challenge at the cellular and organellar level (Apel and Hirt 2004). For example, exposure to cadmium (Cd) is associated with increased ROS generation in plants (Sharma and Dietz 2009, Cuypers *et al*. 2011) and mitochondria in particular (Heyno *et al*. 2008). Moreover, we reported that under Cd exposure, the AOX pathway is activated at transcriptional and translational level in *A. thaliana* (Keunen *et al*. 2013). Nonetheless, the functional implications of AOX induction during Cd stress are still largely unknown.

Similarly to its function, it remains unclear how AOX is induced. Wang *et al*. (2010) have shown that both hydrogen peroxide (H_2O_2) and ethylene are involved in activating alternative respiration in salt-stressed *Arabidopsis* calli. Both ROS (Cuypers *et al*. 2011) and ethylene (Gallego *et al*. 2012, Schellingen *et al*. 2014) are known to mediate Cd stress responses and therefore might also act in the induction and activation of AOX during exposure to Cd.

The current study aims to unravel if and how AOX modulates Cd stress responses in *A. thaliana*. To this end, we compared WT and *aox1a* knockout plants exposed to sublethal Cd concentrations and monitored the Cd-induced oxidative challenge at the transcript and metabolic level in leaves. Moreover, the emerging link between ROS, ethylene and AOX induction and regulation was investigated through a combined reverse genetic approach.

5.2. MATERIALS AND METHODS

5.2.1. Plant culture and cadmium exposure

Wild-type (WT), *aox1a* knockout (SALK_084897 T-DNA insertional line for *AOX1a*; Alonso *et al.* 2003; Watanabe *et al.* 2008), ACC synthase (ACS) *acs2-1/acs6-1* double knockout [N16581 NASC line, defective in Cd-induced ethylene biosynthesis (Schellingen *et al.* 2014)] and ethylene-insensitive *ein2-1* and *ein2-5* (Alonso *et al.* 1999) mutant genotypes were confirmed using PCR as described in the above-mentioned papers. All seeds were surface-sterilised and grown on hydroponics (Smeets *et al.* 2008), except that purified sand was used instead of rock wool (Keunen *et al.,* 2011). A modified Hoagland nutrient solution was used (section 3.2.1., Smeets *et al.* 2008) and growth conditions were set at a 12 h photoperiod, 65% relative humidity and day/night temperatures of 22 °C and 18 °C respectively. Light was provided by Philips Green-Power LED modules. A combination of blue, red and far-red modules was used to obtain a spectrum simulating the photosynthetic active radiation (PAR) of sunlight. The PAR provided at the rosette level was 170 µmol m^{-2} s^{-1}. After 19 days of growth, plants were either exposed to 5 or 10 µM $CdSO_4$ supplied to the roots or further grown under control conditions. After 24 and 72 h, leaf (entire rosette) samples were taken and the fresh weight was determined. Samples were snap frozen in liquid nitrogen and stored at -70 °C for further analyses, except for Cd content determination (cfr. *infra*; Keunen *et al.* 2013).

5.2.2. Determination of Cd and relative water content

During harvest, leaves were rinsed using distilled water. Samples were oven-dried and digested with 70-71% HNO_3 in a heat block (Cuypers *et al*. 2002). Concentrations of Cd were determined via inductively coupled plasma –

optical emission spectrometry (ICP-OES, Agilent Technologies, 700 Series, Belgium). For reference purposes, blank (HNO_3 only) and standard [NIST Spinach (1570a)] samples were used.

Oven-dried samples were weighed to determine the percentage of dry weight per plant, calculated as the ratio between dry and fresh weight. Relative water content per plant was calculated as the ratio between (fresh weight – dry weight) and (saturated weight – dry weight), expressed as a percentage. The saturated weight was fixed at the average weight of the control samples for each genotype per time point.

5.2.3. Gene expression analysis

Frozen leaf samples were disrupted in 2 mL microcentrifuge tubes using two stainless steel beads and the Retsch Mixer Mill MM 400 (Retsch, Belgium) under frozen conditions. From the disrupted tissues, RNA was extracted using the RNAqueous® Total RNA Isolation Kit (Ambion, Life Technologies, Belgium). Concentration and purity of the isolated RNA was assessed using the NanoDrop® ND-1000 spectrophotometer (ThermoScientific, USA). To remove any contaminating genomic DNA, equal amounts (1 µg) of the extracted RNA samples were subjected to a DNase treatment using the TURBO DNA-*free*™ Kit (Ambion). Treated RNA samples were converted to single stranded cDNA via the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Bio Inc., Westburg, the Netherlands). A tenfold dilution of the cDNA was made in 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0) and stored at -20 °C.

Quantitative real-time PCR was performed in optical 96-well plates using the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) and the Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. Amplification occurred at universal cycling conditions (20 s at 95 °C, 40 cycles of 3 s at 95 °C and 30 s at 60 °C) followed by the generation of a dissociation curve to verify amplification specificity. Gene-specific forward and reverse primers (300 nM unless stated otherwise, Supplementary Table 5.1) were designed and optimised via the Primer Express software (v2.0, Applied Biosystems).

Gene expression levels were calculated via the $2^{-\Delta Cq}$ method relative to the sample with the highest expression (minimum Cq). All data were normalised to the expression of three stable reference genes (Remans *et al*. 2008) selected by geNorm (v3.5, Vandesompele *et al*. 2002) and Normfinder (v0.953, Andersen *et al*. 2004) algorithms. Data were normalised using the geometric average of the $2^{-\Delta Cq}$ values for *AT2G28390* (SAND family), *AT4G34270* (TIP41-like) and *AT5G25760* (UBC). All details of our workflow according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines as described by Bustin *et al*. (2009) are shown in Supplementary Table 5.2.

5.2.4. Hierarchical clustering of gene expression data

To identify potential sample-related patterns during Cd exposure in WT versus *aox1a* knockout leaves, hierarchical clustering analysis was performed using GenEx software (v6, MultiD Analyses AB, Sweden). This analysis was based on raw gene expression values and the "Average linkage" algorithm, defining the distance between groups/treatments as the average of distances between all pairs of individuals in all groups. Distances between the measures were calculated via the Euclidian Distance Measure. Heat maps were constructed to compare expression levels between different genes and samples.

5.2.5. In situ detection of H_2O_2 using 3,3'-diaminobenzidine

Leaves were stained using 3,3'-diaminobenzidine (DAB) as described by Daudi *et al*. (2012). After DAB oxidation by H_2O_2, an insoluble brown precipitate reflects the presence and tissue distribution of H_2O_2. Leaves (three per plant, six biological replicates per condition) were incubated in 3 mL freshly prepared staining solution [DAB (1 mg mL^{-1}) and Tween-20 (0.05% v/v) in 10 mM Na_2HPO_4, pH 3.0] or control solution (10 mM Na_2HPO_4) using 12-well microtiter plates. As DAB is light-sensitive, all plates were covered using aluminium foil. To improve DAB infiltration into the leaves, vacuum was applied during 5 min using a desiccator. After shaking the plates at 80 rpm for 4 h, all solutions were replaced by 3 mL bleaching solution (ethanol:acetic acid:glycerol; 3:1:1 v:v:v). After incubation at 95 °C during 15 min, the bleaching solution was refreshed, allowed to incubate at room temperature for 30 min and finally stored at 4 °C.

The following day, leaves were visualised under white light using a binocular microscope. Photographs were obtained using a digital camera and BTV-pro software (Bensoftware). Experiments were repeated twice using independent biological replicates and representative pictures are depicted.

5.2.6. Determination of glutathione content and redox state

Contents of oxidised (GSSG) and reduced (GSH) forms of glutathione were spectrophotometrically determined using the plate reader method previously described by Queval and Noctor (2007). Frozen leaf samples (100 mg) were thoroughly ground in liquid nitrogen using a cooled mortar and pestle. Sample powders were homogenised in 200 mM HCl (800 µL per 120 mg fresh sample weight) and centrifuged during 10 min (16 000 *g*, 4 °C). Samples were adjusted to pH 4.5 and kept at 4 °C during the entire procedure unless specifically mentioned otherwise. Measurement of GSH and GSSG is based on the reduction of 5,5-dithiobis(2-nitro-benzoic acid) (DTNB, 600 µM) by the action of glutathione reductase (GR, 1U mL^{-1}) in the presence of NADPH (500 µM), which was spectrophotometrically monitored at 412 nm during 5 min. Total glutathione concentrations (GSH + GSSG) were calculated relative to a standard curve ranging from 0 to 500 pmol GSH. To determine oxidised GSSG amounts, samples were first incubated with 2-vinyl-pyridine (2-VP, 1% v/v) to precipitate all free GSH present in the sample during 30 min at room temperature. Samples were centrifuged twice (16 000 *g*, 4 °C) to precipitate 2-VP prior to the measurement. For quantification purposes, a GSSG standard curve ranging from 0 to 100 pmol was incubated with 2-VP and measured in duplicate concurrently with the samples. Reduced GSH concentrations were derived by subtracting oxidised GSSG from total levels (Queval and Noctor 2007).

5.2.7. Statistical analyses

All datasets were statistically analysed with ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparisons using R version 2.13.1 (R Development Core Team 2011). Both normality and homoscedasticity were checked; transformations were applied when necessary to approximate normality. If normality could not be reached, a non-parametric Kruskal-Wallis test, followed by the Wilcoxon rank sum test was used to determine statistical

significance of the data. Outliers were determined using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05. The statistical analysis used is indicated in the caption of each Table or Figure.

For gene expression data, normalised relative quantities were log transformed prior to statistical analysis (Supplementary Table 5.2). Both differences within and between genotypes (Supplementary Table 5.3) and overall genotype * treatment interaction effects (Supplementary Table 5.4) are discussed per time point. Significant interaction effects depict genes where treatment (Cd) effects differ between both genotypes.

5.3. RESULTS

Responses of WT versus *aox1a* knockout plants were compared after 24 and 72 h of exposure to 5 or 10 µM Cd. Both concentrations were previously demonstrated to be sublethal for the WT (Keunen *et al*. 2011). The two time points were selected based on our kinetic study in Cd-exposed WT plants, where *AOX1a* expression peaked after 24 h and was still enhanced after 72 h Cd exposure in the leaves (Keunen *et al*. 2013).

5.3.1. Growth parameters and Cd uptake

After 24 h, no significant changes in leaf fresh weight could be observed for WT or *aox1a* knockout plants (Table 5.1A and B). Significant decreases in fresh weight as compared to leaves of unexposed plants were only apparent after 72 h of Cd exposure in both genotypes (Table 5.1A and B). In general, changes in leaf fresh weight were mirrored by the impact of Cd on dry weight percentages (Table 5.1C) and relative water content (Table 5.1D) in a dose-dependent manner after 72 h in both genotypes.

The rise in Cd content in the leaves was correlated with the Cd concentration applied to the nutrient solution, without any significant differences depending on the genotype (Table 5.2).

Table 5.1. *Weight parameters of leaves harvested from wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants. All parameters were determined for 19-days-old plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 5 biological replicates, each consisting of at least 10 individual rosettes. (A) Fresh weight expressed in mg per plant. (B) The percentage of growth inhibition relative to the control (0.00%). (C) Percentage dry weight per plant. (D) Relative water content per plant. Different letters represent significant differences within and between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA).*

	24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***
A. Fresh weight (mg)				
Control	60.08 ± 2.31a	55.12 ± 2.97a	100.84 ± 2.94a	89.98 ± 4.64a
5 µM Cd	57.20 ± 2.62a	55.70 ± 0.72a	73.45 ± 4.28b	67.06 ± 3.01b,d
10 µM Cd	54.30 ± 2.76a	56.36 ± 1.84a	46.93 ± 3.22c	52.69 ± 2.48c,d
B. Growth inhibition relative to control (%)				
Control	0.00a	0.00a	0.00a	0.00a
5 µM Cd	4.79a	0.00a	27.16b,d	25.47b
10 µM Cd	9.61a	0.00a	53.46c	41.44c,d
C. Dry weight (%)				
Control	8.95 ± 0.12a	8.85 ± 0.13a	8.73 ± 0.08a	8.74 ± 0.07a
5 µM Cd	9.35 ± 0.18a,b	9.30 ± 0.09a,b	10.82 ± 0.36b	10.90 ± 0.32b
10 µM Cd	9.83 ± 0.11b	9.85 ± 0.12b	13.99 ± 0.60c	13.68 ± 0.58c
D. Relative water content (%)				
Control	100.08 ± 4.19a	100.11 ± 5.91a	100.03 ± 3.20a	100.11 ± 5.66a
5 µM Cd	94.80 ± 4.78a	101.17 ± 1.43a	70.56 ± 4.51b,d	72.31 ± 3.60b
10 µM Cd	89.56 ± 4.98a	102.57 ± 3.74a	42.88 ± 3.18c	54.99 ± 2.86c,d

Table 5.2. *Cadmium content (mg kg^{-1} dry weight) of leaves harvested from wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants. Cadmium levels were determined in 19-days-old plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 5 biological replicates. No Cd could be detected in unexposed plants (nd). Different letters represent significant differences within and between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA).*

	24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***
Control	nd	nd	nd	nd
5 µM Cd	799.25 ± 47.01a	803.66 ± 24.94a	1440.93 ± 78.58a	1578.63 ± 61.63a
10 µM Cd	1441.17 ± 115.12b	1284.11 ± 117.28b	2027.68 ± 138.17b	2273.63 ± 106.61b

5.3.2. Connection between AOX1a and the Cd-induced oxidative challenge in Arabidopsis thaliana leaves

When comparing WT and *aox1a* knockout plants, it is worthwhile to note that transcript levels of all measured genes (except for *AOX1a*) did not differ between unexposed genotypes. We previously demonstrated that sublethal Cd

exposure activates the mitochondrial alternative ETC at the transcript level in *A. thaliana* WT leaves. In this research, it was demonstrated that only *AOX1a* and *AOX1d* are expressed and increased upon Cd exposure in the leaves under our experimental conditions (Chapter 4, Keunen *et al*. 2013). In the present study, *AOX1a* transcript levels significantly peaked after 24 h exposure to both Cd concentrations in the WT (Figure 5.1A). Without functional AOX1a, transcript levels of *AOX1d* augmented to a greater extent after prolonged (72 h) exposure to 5 and 10 µM Cd in the leaves as compared to the WT (Figure 5.1B).

Although genotype-dependent differences were observed for *AOX1a* and *AOX1d* expression, Cd-induced changes in expression of alternative ETC components such as alternative NAD(P)H dehydrogenases (NDs) and uncoupling proteins (UCPs) (Keunen *et al*. 2013) were generally similar in both WT and *aox1a* knockout leaves (Supplementary Table 5.3). However, a significant overall genotype * treatment interaction effect could be observed for *UCP5* after 72 h (Supplementary Table 5.4), indicating that the treatment effect differs between both genotypes. Indeed, expression levels of this gene were higher in *aox1a* knockout than in WT leaves from plants exposed to 5 µM Cd during 72 h (Supplementary Table 5.3).

Gadjev *et al*. (2006) have described a set of five transcripts that were upregulated more than fivefold in different experimental conditions eliciting oxidative stress. These genes are now collectively referred to as hallmarks for general oxidative stress, independent of the ROS type or where ROS are produced (Gadjev *et al*. 2006). Transcript levels of all hallmark genes were increased in WT and *aox1a* knockout plants upon Cd exposure (Figure 5.1C-G). It should be noted however that overall genotype * treatment interaction effects were significant for three markers after 72 h (Supplementary Table 5.4). Transcript levels of the "*up*regulated by *ox*idative stress" gene (*UPOX*, *AT2G21640*), expressed in plant mitochondria (Sweetlove *et al*. 2002), were used to estimate the extent of the mitochondrial oxidative challenge induced by Cd. A clear genotype-dependent effect was detected as *UPOX* expression was upregulated to a higher level after 72 h exposure to 5 µM Cd in *aox1a* knockout in comparison to WT leaves (Figure 5.1C). Similar results were obtained for the unknown *AT1G19020* (Figure 5.1E) and TIR-class gene (*AT1G57630*, Figure 5.1G).

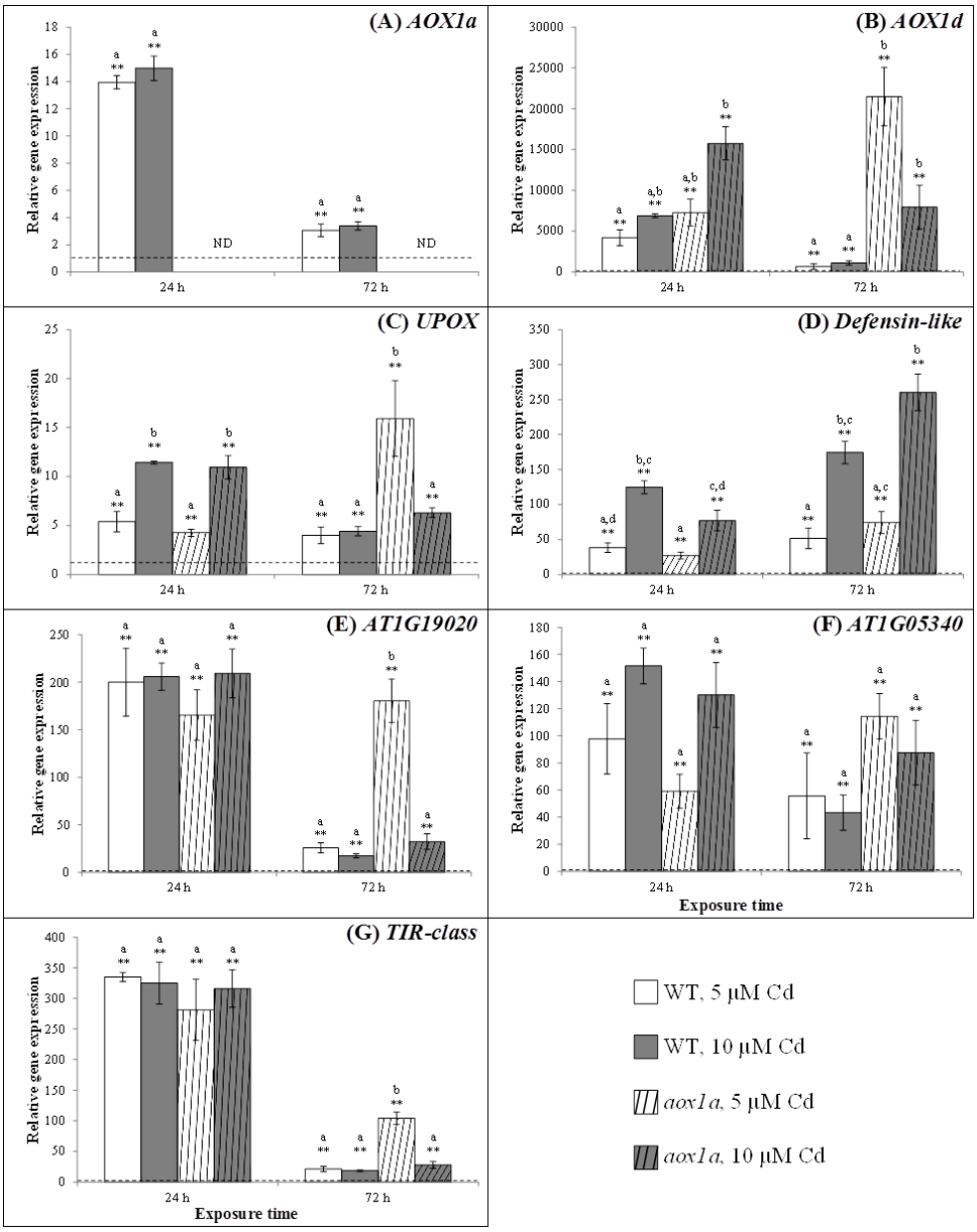


Figure 5.1. *Relative leaf transcript levels of AOX1a (A), AOX1d (B) and the oxidative stress hallmark genes (C-G) in Arabidopsis thaliana. Transcript levels were measured via quantitative real-time PCR in leaf samples of 19-days-old wild-type (WT) and aox1a knockout (aox1a) plants exposed to 5 µM (WT: white; aox1a: white striped) or 10 µM (WT: grey; aox1a: grey striped) $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks: ** p < 0.01.*

Figure 5.1 (continued). *Different letters denote significant differences within and between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA). AOX, alternative oxidase; UPOX, upregulated by oxidative stress; TIR, Toll-Interleukin-1.*

To further characterise the Cd-induced oxidative challenge at the metabolic level, H_2O_2 production and glutathione (GSH) concentrations and redox state were determined. In both genotypes, Cd exposure increased the presence of DAB precipitates after 24 and 72 h (Figure 5.2). However, the leaves of *aox1a* knockout plants exposed to 5 µM Cd showed less intense staining as compared to WT plants (Figure 5.2, middle row panels), pointing towards a lower production of H_2O_2 under these conditions. Concerning the concentrations and redox state of GSH (Table 5.3), a marked decline in GSSG content was observed for WT and *aox1a* knockout plants after 24 h exposure to 5 or 10 µM Cd. Consequently, a more reduced redox state was apparent (Table 5.3). After 72 h, GSSG levels rose at 10 µM Cd, however only significantly in *aox1a* knockout mutants, but without significant alteration of the redox state (Table 5.3). Reduced and total GSH levels significantly increased in 5 µM Cd-exposed WT plants after 72 h. This was not observed in *aox1a* knockout mutants (Table 5.3). However, exposure to 10 µM Cd elicited similar increases in GSH levels in both WT and *aox1a* knockout leaves (Table 5.3).

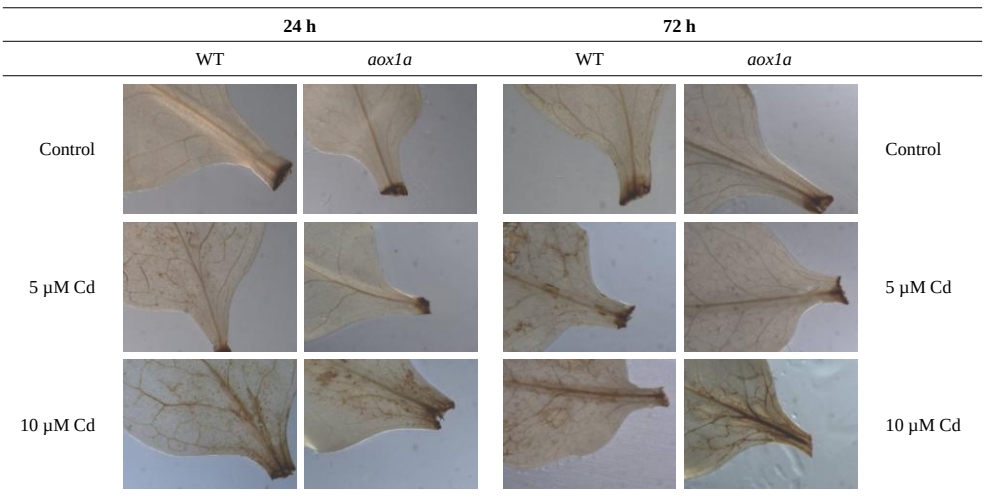


Figure 5.2. *Hydrogen peroxide (H_2O_2) production as detected by 3,3'-diaminobenzidine (DAB) in the leaves. Staining was performed using leaves of wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants either exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per condition, 3 rosette leaves of 6 biological replicates were sampled, and representative photographs from 2 independent experiments are depicted here.*

Table 5.3. *Leaf glutathione (GSH) concentrations (nmol GSH equivalents g^{-1} fresh weight) and redox state. Total GSH levels consist of both reduced (GSH) and oxidised glutathione disulfide (GSSG), thereby also determining the ratio between oxidised and reduced forms i.e. the redox state. Concentrations were determined in leaves of 19-days-old wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of at least 4 biological replicates. Per time point, significant Cd-induced changes within a genotype are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for increases and decreases respectively (one-way ANOVA).*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Total GSH + GSSG	**Control**	223.53 ± 12.86	210.48 ± 15.78	171.03 ± 9.31	173.22 ± 5.82
	5 µM Cd	194.31 ± 25.34	201.80 ± 13.37	312.34 ± 25.31	254.95 ± 29.83
	10 µM Cd	257.82 ± 15.06	218.30 ± 16.92	397.90 ± 43.90	456.04 ± 36.92
GSH	**Control**	216.76 ± 13.10	197.37 ± 15.33	162.39 ± 9.50	166.03 ± 5.98
	5 µM Cd	193.51 ± 25.39	197.47 ± 12.04	305.14 ± 24.42	246.69 ± 30.30
	10 µM Cd	255.14 ± 14.50	213.39 ± 17.01	381.01 ± 43.94	445.86 ± 36.00
GSSG	**Control**	6.77 ± 1.60	13.10 ± 1.51	8.64 ± 1.41	5.15 ± 0.65
	5 µM Cd	0.80 ± 0.11	0.94 ± 0.20	7.20 ± 1.66	8.26 ± 1.13
	10 µM Cd	2.68 ± 1.22	4.91 ± 1.02	16.89 ± 4.41	10.18 ± 1.31
GSSG / GSH	**Control**	0.032 ± 0.008	0.067 ± 0.008	0.054 ± 0.010	0.031 ± 0.005
	5 µM Cd	0.005 ± 0.001	0.005 ± 0.001	0.023 ± 0.005	0.037 ± 0.008
	10 µM Cd	0.010 ± 0.005	0.024 ± 0.006	0.048 ± 0.013	0.023 ± 0.002

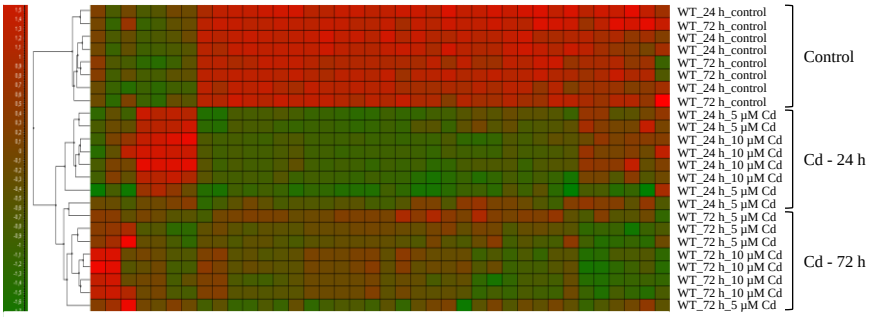
5.3.3. Transcriptional alterations in ROS producing and scavenging components

Exposure to Cd impacts on transcript levels of genes encoding ROS producing and scavenging proteins (Cuypers *et al*. 2011, Jozefczak *et al*. 2014). In this study, our aim was to determine if and how AOX1a affects the Cd-induced oxidative challenge at the transcript level in *A. thaliana* leaves. Therefore, relative expression levels of different ROS producing, both mitochondrial and other ROS scavenging genes (Supplementary Table 5.1) were determined in WT and *aox1a* knockout plants exposed to 5 or 10 µM Cd during 24 and 72 h (Supplementary Tables 5.3 and 5.4). In general, for WT plants, responses coincided with our earlier work as described by Cuypers *et al*. (2011), Keunen *et al*. (2013) and Jozefczak *et al*. (2014). Expression levels of ROS producing lipoxygenases and antioxidative genes such as catalase isoforms 1 and 3 and glutathione reductase 1 were increased after Cd exposure. Moreover, the Cd-mediated decrease in copper/zinc superoxide dismutase expression was confirmed (Supplementary Table 5.3). When comparing WT and *aox1a* knockout plants, it is worthwhile to note that transcript levels of all measured genes did

not differ between unexposed genotypes. Although Cd-induced expression changes were generally similar in both genotypes, a significant genotype * treatment interaction effect was observed for the respiratory burst oxidase homologue C (*RBOHC*) gene after prolonged (72 h) Cd exposure (Supplementary Table 5.4). Indeed, *RBOHC* expression levels increased to a greater extent in *aox1a* knockout as compared to WT plants after 72 h exposure to 5 µM Cd (Supplementary Table 5.3).

Hierarchical clustering analysis including all samples and all measured genes (Supplementary Table 5.1) was performed for both genotypes (Figure 5.3). Control samples clustered apart from Cd-exposed samples in WT as well as in *aox1a* mutant plants. In WT leaves, Cd-exposed samples were generally grouped per time point (24 and 72 h) independent on the Cd concentration applied (Figure 5.3A). While the same held true for Cd-exposed samples after 24 h in *aox1a* knockout mutants, a separate clustering of 5 and 10 µM Cd-exposed samples occurred after 72 h in the absence of AOX1a (Figure 5.3B).

A



B

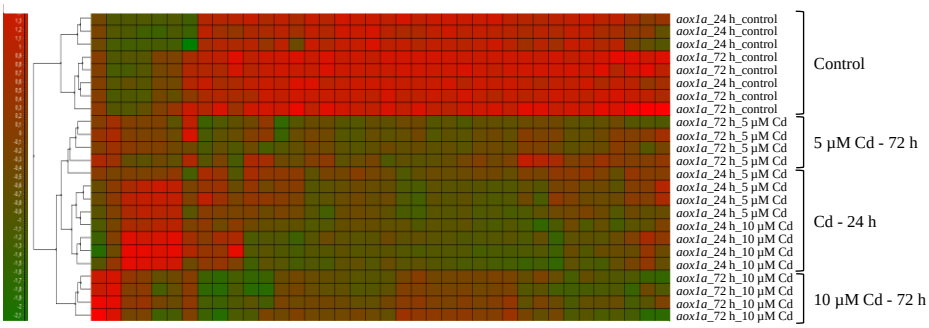


Figure 5.3. *Hierarchical classification of transcript levels in leaf samples. Results are visualised using dendrograms and heat maps indicating expression levels in the leaves of wild-type (A) and aox1a knockout (B) Arabidopsis thaliana plants. Each column of the map represents a different gene.*

5.3.4. Mechanistic insights into AOX induction and regulation in Cd-exposed Arabidopsis thaliana leaves

Ethylene is undoubtedly involved in Cd-induced signalling as recently evidenced by Schellingen *et al*. (2014). Moreover, ethylene and/or ROS are potentially involved in modulating AOX upregulation during stress (Wang *et al*. 2010, Li *et al*. 2013). To further strengthen the emerging link between ethylene, ROS and AOX, we used a reverse genetic approach combining ethylene biosynthesis (*acs2-1/6-1* knockout), ethylene signalling (*ein2-1*) and *aox1a* knockout mutants. Based on the increased induction of the oxidative stress hallmark genes in leaves of 5 µM Cd-exposed *aox1a* knockout as compared to WT plants, all genotypes were exposed to 5 µM Cd during 24 and 72 h. Transcript levels were compared between different genotypes and expressed relatively to their own control (Figure 5.4). Under control conditions, expression levels of *AOX1a*, *AOX1d* and *RBOHC* were significantly higher in *ein2-1* mutants as compared to the WT, albeit only after 24 h (Supplementary Table 5.5). Upregulation of *AOX1a* (Figure 5.4A) and *AOX1d* (Figure 5.4B) significantly declined in leaves of both ethylene mutants exposed to 5 µM Cd as compared to WT plants. Moreover, *RBOHC* upregulation was diminished or even absent in Cd-exposed *acs2-1/6-1* and *ein2-1* mutants (Figure 5.4C). Similar results were obtained for the leaves of 5 µM Cd-exposed *ein2-5* mutants (Supplementary Figure 5.1).

After 72 h exposure to 5 µM Cd, transcript levels of *RBOHC* were higher in *aox1a* knockout leaves as compared to the WT (Figure 5.4D). Similarly, expression of the ethylene biosynthesis ACC synthase 6 (*ACS6*, Figure 5.4E) and the ethylene receptor 2 (*ETR2*, Figure 5.4F) gene was induced to a greater extent under the same conditions. Both observations point towards a regulatory role of AOX1a in the upregulation of *RBOHC* and genes involved in ethylene synthesis and signalling (middle scheme in Figure 5.4). For the sake of completeness, transcript levels of ethylene biosynthesis as well as signal transduction genes (Supplementary Table 5.1) were determined in WT and *aox1a* knockout plants, also after exposure to 10 µM Cd (Supplementary Table 5.3). In general, most differences observed between both genotypes after prolonged (72 h) exposure to 5 µM Cd (Figure 5.4) disappeared after exposure to the highest Cd concentration (Supplementary Table 5.3).

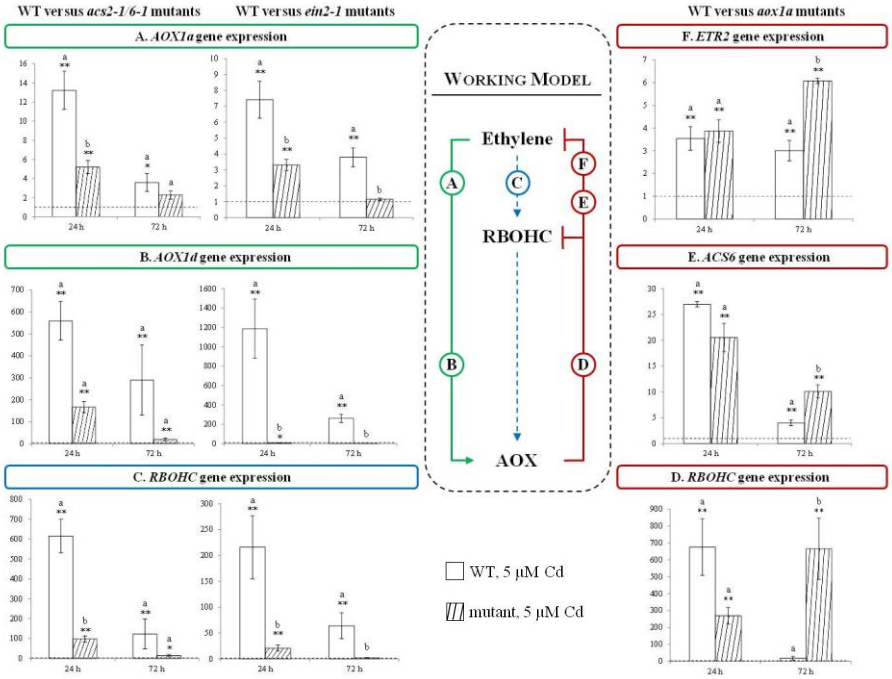


Figure 5.4. *Schematic overview of the interplay between ethylene, RBOHC and AOX in leaves of Cd-exposed Arabidopsis thaliana plants. Transcript levels were measured via quantitative real-time PCR in leaf samples of 19-days-old wild-type (WT) plants and acs2-1/acs6-1 double knockout (acs2-1/6-1), ethylene-insensitive ein2-1 or aox1a knockout mutants (aox1a) exposed to 5 µM $CdSO_4$ (WT: white; mutant: white striped) during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks: * $p < 0.05$, ** $p < 0.01$. Different letters denote significant differences between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA). Expression levels of AOX1a (A), AOX1d (B) and RBOHC (C) were determined in WT and acs2-1/6-1 and ein2-1 mutants. Moreover, RBOHC (D), ACS6 (E) and ETR2 (F) transcript levels were measured in WT and aox1a mutants. A working model for the putative interactions between ethylene, RBOHC and AOX is depicted in the middle. ACS, ACC synthase; AOX, alternative oxidase; ETR, ethylene receptor; RBOHC, respiratory burst oxidase homologue C.*

5.4. Discussion

Previous results have indicated that AOX respiration is involved in *A. thaliana* stress responses to Cd at transcript and protein level (Keunen *et al*. 2013). In the current work, we studied the significance of AOX induction during moderate (5 µM) and more severe (10 µM) Cd stress by comparing WT and *aox1a* knockout plants. Prior to Cd exposure, no clear phenotypic differences were observed between both genotypes. Coinciding with previous research (Giraud

et al. 2008), this supports the view that a lack of AOX has minor to no consequences under non-stress conditions but particularly impacts (abiotic) stress responses (Vanlerberghe *et al*. 2009). However, Cd content and weight parameters were similarly affected in WT and *aox1a* knockout plants (Tables 5.1 and 5.2). Therefore, Cd-induced responses were compared between both genotypes at the molecular level.

5.4.1. A modulating role for AOX1a during the oxidative challenge under moderate (5 µM) Cd exposure

In general, our assessment of the Cd-induced oxidative challenge at transcript and metabolic level revealed most differences between WT and *aox1a* knockout plants under moderate *i.e.* 5 µM Cd exposure. For example, expression levels of the oxidative stress hallmark genes *UPOX* (Figure 5.1C), *AT1G19020* (Figure 5.1E) and the TIR-class gene (Figure 5.1G) were induced to a higher extent after 72 h exposure to 5 µM Cd in *aox1a* knockout in comparison to WT leaves. As demonstrated by Cvetkovska and Vanlerberghe (2012), a lack of AOX increases mitochondrial $O_2^{\bullet-}$ production. Therefore, higher *UPOX* upregulation in the absence of AOX1a coincides with an enhanced mitochondrial oxidative challenge under moderate Cd stress. Moreover, a lack of functional AOX1a can have consequences outside mitochondria, as evidenced by the higher upregulation of the chloroplast TIR-class gene in 5 µM Cd-exposed *aox1a* knockout versus WT leaves. Similarly, Giraud *et al*. (2008) reported increases in stress-responsive transcripts and particularly those that encode ROS scavengers in chloroplasts during combined light and drought stress in the absence of AOX1a.

Under normal conditions, it has been shown that AOX-suppressed tobacco leaves have similar to lower H_2O_2 concentrations as compared to WT leaves (Cvetkovska and Vanlerberghe 2012). Consistently, results in both tobacco (Amirsadeghi *et al*. 2006) and *Arabidopsis* (Watanabe *et al*. 2008) point towards induced ROS scavenging when AOX is missing. On the contrary, we did not observe differences in DAB staining between WT and *aox1a* knockout leaves under normal growth conditions. During exposure to 5 µM Cd however, H_2O_2 accumulated to a lesser extent in *aox1a* knockout leaves (Figure 5.2). Pasqualini *et al*. (2007) reported that leaves of transgenic tobacco plants overexpressing

AOX1a showed increased and persistent H_2O_2 levels during acute ozone fumigation as compared to WT plants. The attenuation of H_2O_2 levels after 24 and 72 h of Cd exposure in our study could have an impact at a more prolonged stage (72 h), for example when comparing GSH responses in both genotypes. The absence of increased GSH levels in leaves of 5 µM Cd-exposed *aox1a* knockout as compared to WT plants after 72 h (Table 5.3) coincides with an altered oxidative challenge as evidenced by the hallmark genes (Figure 5.1).

Taken together, our data suggest that a lack of functional AOX1a disrupts a signalling pathway emerging from the mitochondrion early after the start of Cd exposure. A role for AOX1a in acute Cd stress responses is underlined by the observed peak in its expression levels after 24 h (Figure 5.1A) and its transient increase at the protein level (Keunen *et al*. 2013). The nature of the retrograde signal initiated by AOX is currently unknown, but ROS are often put forward (Vanlerberghe *et al*. 2009). In line with this, we suggest the involvement of H_2O_2 as its levels were attenuated in leaves of plants lacking AOX1a during 5 µM Cd exposure. Nevertheless, the role of other signalling metabolites should also be explored as AOX is intimately related to the carbon and nitrogen metabolism under physiological (Gandin *et al*. 2014) and stress conditions (Watanabe *et al*. 2008). In addition, it is unclear which cellular functional level is controlled by the AOX1a signalling pathway during Cd stress. Expression levels of genes involved in the ROS network did not change in leaves of *aox1a* knockout versus WT plants (Supplementary Table 5.3). As GSH responses did alter in the absence of AOX1a, we suggest the AOX1a signalling pathway to regulate defence mechanisms to moderate (5 µM) Cd stress at a post-transcriptional level in *A. thaliana* leaves.

In reverse genetic studies, it is often observed that other components of the alternative respiratory pathway such as NDs and UCPs try to compensate for the absence of functional AOX1a. For example, Watanabe *et al*. (2008) have demonstrated increased transcript levels of *NDB2* and *UCP1* isoforms in *A. thaliana* leaves lacking AOX1a as compared to the WT under low temperature. While in our study *ND* and *UCP* genes mostly did not show altered expression levels in the absence of AOX1a (Supplementary Table 5.3), expression of *AOX1d* was more abundant in Cd-exposed *aox1a* knockout leaves, again highly pronounced after 72 h exposure to 5 µM Cd (Figure 5.1B). Strödtkotter *et al*.

(2009) have demonstrated that AOX1d was unable to take over the function of AOX1a in *A. thaliana* exposed to antimycin A. Functional compensation of AOX1a by AOX1d is unlikely in our conditions as well, since *aox1a* knockout mutants did show a differential response to 5 µM Cd as compared to WT plants.

Finally, all differences observed in leaves of 5 µM Cd-exposed *aox1a* knockout as compared to WT plants after 72 h disappeared when comparing responses to the highest Cd concentration. This is supported by our clustering analysis, where samples from 5 µM Cd-exposed mutant leaves clustered separately from those derived from plants exposed to 10 µM Cd at 72 h (Figure 5.3). The latter plants severely suffer as indicated by approximately 50% growth inhibition (Table 5.1B), extensive water loss (Table 5.1D) and increased total H_2O_2 levels (Figure 5.2). The enhanced ROS accumulation did cause oxidative damage, but only after 72 h exposure to the highest Cd concentration (Keunen *et al*. 2013). Therefore, we hypothesise the AOX1a signal to initiate a fine-tuning pathway that is overwhelmed when stress levels are more severe (10 µM Cd). Nonetheless, plants are able to survive long-term exposure to 10 µM Cd (Keunen *et al*. 2011).

5.4.2. The emerging link between ethylene, ROS and AOX in Cd-exposed Arabidopsis thaliana leaves

Recently, more research is devoted to unravelling the nature of primary signals that genetically control AOX respiration in plants (Vanlerberghe 2013). Li *et al*. (2013) have shown that mitochondrial $O_2^{\bullet-}$ modulates rice *AOX1* gene expression under cold, drought and salinity stress. In addition to ROS, ethylene has been implicated in the induction of alternative respiration in salt-treated *Arabidopsis* calli (Wang *et al*. 2010).

In the current work, we demonstrate the necessity of functional ethylene biosynthesis and signal transduction to fully induce *AOX1* genes in leaves of 5 µM Cd-exposed *A. thaliana* plants (Figure 5.4). To this end, we compared transcriptional responses in Cd-exposed WT and *acs2-1/6-1* knockout plants. The use of these mutants is justified as increased expression levels of both *ACS2* and *ACS6* mainly mediate ethylene biosynthesis and responses in Cd-exposed *A. thaliana* plants (Schellingen *et al*. 2014). In *acs2-1/6-1* knockout mutant leaves, induction of both *AOX1a* and *AOX1d* was lowered as compared to the

WT, mostly pronounced after 24 h exposure to 5 µM Cd. Furthermore, Cd-induced responses were compared between WT plants and *ein2-1* mutants. As EIN2 is a central component in the ethylene signalling pathway, *ein2-1* mutant lines are completely insensitive to this hormone (Alonso *et al*. 1999). Under ethylene insensitive conditions, the induction of *AOX1a* and *AOX1d* by Cd completely disappeared after 72 h (Figure 5.4A and B). A similar response was observed for *ein2-5* mutants as compared to the WT (Supplementary Figure 5.1). A link between ethylene and *AOX1d* induction was also reported by Buchanan-Wollaston *et al.* (2005), who observed diminished upregulation of *AOX1d* in senescing leaves of *ein2-1* mutants as compared to WT plants. Furthermore, AOX was demonstrated to play a regulatory role during ethylene-induced plant cell death (Lei *et al*. 2003) as well as tomato fruit ripening (Xu *et al*. 2012). Exposure to Cd is related to accelerated leaf ageing (Sandalio *et al.* 2001) and ethylene might at least contribute to this response in *A. thaliana* (Schellingen *et al*. 2014).

Although ethylene induces *AOX1* expression during Cd exposure, other components are suggested to be involved. Ederli *et al*. (2006) showed that both ethylene-dependent and -independent pathways are required to increase *AOX1a* expression in tobacco plants exposed to ozone. In addition, evidence was presented for the essential role of nitric oxide (NO) as upstream signalling component activating *AOX*. As NO is suggested to be involved in Cd stress responses as well (Arasimowicz-Jelonek *et al*. 2011), its role in genetic control of AOX respiration should be addressed in future studies.

It is tempting to speculate the involvement of ROS and more particularly $O_2^{\bullet-}$ generated by NADPH oxidases (dashed arrows in Figure 5.4) in the modulation of *AOX* by ethylene. Jiang *et al.* (2013) have demonstrated that enhanced ethylene production potently promotes salt tolerance in *A. thaliana*, which is correlated with elevated ROS levels and RBOHF function. Moreover, expression of *RBOHD* and *RBOHF* genes is preceded by ethylene biosynthesis in *Brassica oleracea* (Jakubowicz *et al*. 2010). Upon Cd exposure, expression of *RBOHC* that was the highest induced isoform in leaves (Remans *et al*., 2010) did not increase to WT levels in *acs2-1/6-1* knockout, *ein2-1* (Figure 5.4C) and *ein2-5* mutants (Supplementary Figure 5.1). These results clearly link ethylene to ROS production by NADPH oxidases, which might also mediate stress responses

inducing *AOX1* genes and leading to signalling and acclimation during moderate *i.e.* 5 µM Cd exposure. On the other hand, the involvement of negative feedback mechanisms from AOX to RBOHC/ethylene was demonstrated using *aox1a* knockout plants. Indeed, the induction of *RBOHC* (Figure 5.4D), the ethylene biosynthesis gene *ACS6* (Figure 5.4E) and ethylene signalling gene *ETR2* (Figure 5.4F) was enhanced in the absence of AOX1a. Taken together, these data suggest a reciprocal crosstalk between ethylene, RBOHC and AOX during moderate Cd exposure in *A. thaliana* leaves (Figure 5.4).

REFERENCES

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*, **64**, 5245–5250.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J.R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science*, **284**, 2148–2152.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C. and Ecker J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Amirsadeghi, S., Robson, C.A., McDonald, A.E. and Vanlerberghe, G.C. (2006) Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signaling molecules. *Plant Cell Physiol*, **47**, 1509–1519.

Apel, K. and Hirt, H. (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol*, **55**, 373–399.

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J. and Gwóźdź, E.A. (2011) The message of nitric oxide in cadmium challenged plants. *Plant Sci*, **181**, 612–620.

Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., Lim, P.O., Nam, H.G., Lin, J.F., Wu, S.H., Swidzinski, J., Ishizaki, K. and Leaver, C.J. (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. *Plant J*, **42**, 567–585.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: Minimum Information for publication of Quantitative real-time PCR Experiments. *Clin Chem*, **55**, 611–622.

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A. and Whelan, J. (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol Biol*, **58**, 193–212.

Cuypers, A., Vangronsveld, J. and Clijsters, H. (2002) Peroxidases in roots and primary leaves of *Phaseolus vulgaris*. Copper and zinc phytotoxicity: a comparison. *J Plant Physiol*, **159**, 869–876.

Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J. and Vangronsveld, J. (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J Plant Physiol*, **168**, 309–316.

Cvetkovska, M. and Vanlerberghe, G.C. (2012) Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol*, **195**, 32–39.

Daudi, A., Cheng, Z., O'Brien, J.A., Mammarella, N., Khan, S., Ausubel, F.M. and Bolwell, G.P. (2012) The apoplastic oxidative burst peroxidase in *Arabidopsis* is a major component of pattern-triggered immunity. *Plant Cell*, **24**, 275–287.

Ederli, L., Morettini, R., Borgogni, A., Wasternack, C., Miersch, O., Reale, L., Ferranti, F., Tosti, N. and Pasqualini, S. (2006) Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants. *Plant Physiol*, **142**, 595–608.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R. and Van Breusegem, F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiol*, **141**, 436–445.

Gallego, S.M., Pena, L.B., Barcia, R.A., Azpilicueta, C.E., Iannone, M.F., Rosales, E.P., Zawoznik, M.S., Groppa, M.D. and Benavides, M.P. (2012) Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environ Exp Bot*, **83**, 33–46.

Gandin, A., Denysyuk, M. and Cousins, A.B. (2014) Disruption of the mitochondrial alternative oxidase (AOX) and uncoupling protein (UCP) alters rates of foliar nitrate and carbon assimilation in *Arabidopsis thaliana*. *J Exp Bot*, **65**, 3133–3142.

Giraud, E., Ho, L.H.M., Clifton, R., Carroll, A., Estavillo, G., Tan, Y.F., Howell, K.A., Ivanova, A., Pogson, B.J., Millar, A.H. and Whelan, J. (2008) The absence of ALTERNATIVE OXIDASE1a in Arabidopsis results in acute sensitivity to combined light and drought stress. *Plant Physiol*, **147**, 595–610.

Heyno, E., Klose, C. and Krieger-Liszkay, A. (2008) Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. *New Phytol*, **179**, 687–699.

Jakubowicz, M., Gałgańska, H., Nowak, W. and Sadowski, J. (2010) Exogenously induced expression of ethylene biosynthesis, ethylene perception, phospholipase D, and Rboh-oxidase genes in broccoli seedlings. *J Exp Bot*, **61**, 3475–3491.

Jiang, C., Belfield, E.J., Cao, Y., Smith, J.A.C. and Harberd, N.P. (2013) An *Arabidopsis* soil- salinity-tolerance mutation confers ethylene-mediated enhancement of sodium/potassium homeostasis. *Plant Cell*, **25**, 3535–3552.

Jozefczak, M., Keunen, E., Schat, H., Bliek, M., Hernández, L.E., Carleer, R., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2014) Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. *Plant Physiol Biochem*, **83**, 1–9.

Keunen, E., Truyens, S., Bruckers, L., Remans, T., Vangronsveld, J. and Cuypers, A. (2011) Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged? *Plant Physiol Biochem*, **49**, 1084–1091.

Keunen, E., Jozefczak, M., Remans, T., Vangronsveld, J. and Cuypers, A. (2013) Alternative respiration as a primary defence during cadmium-induced mitochondrial oxidative challenge in *Arabidopsis thaliana*. *Environ Exp Bot*, **91**, 63–73.

Lei, X.Y., Zhu, R.Y., Zhang, G.Y. and Dai, Y.R. (2003) Possible involvement of the mitochondrial alternative pathway in ethylene-induced apoptosis in tomato protoplasts. *Plant Growth Regul*, **41**, 111–116.

Li, C.R., Liang, D.D., Li, J., Duan, Y.B., Li, H., Yang, Y.C., Qin, R.Y., Li, L., Wei, P.C. and Yang, J.B. (2013) Unravelling mitochondrial retrograde regulation in the abiotic stress induction of rice *ALTERNATIVE OXIDASE 1* genes. *Plant Cell Environ*, **36**, 775–788.

Millar, A.H., Whelan, J., Soole, K.L. and Day, D.A. (2011) Organization and regulation of mitochondrial respiration in plants. *Annu Rev Plant Biol*, **62**, 79–104.

Pasqualini, S., Paolocci, F., Borgogni, A., Morettini, R. and Ederli, L. (2007) The overexpression of an alternative oxidase gene triggers ozone sensitivity in tobacco plants. *Plant Cell Environ*, **30**, 1545–1556.

Purvis, A.C. and Shewfelt, R.L. (1993) Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiol Plant*, **88**, 712–718.

Queval, G. and Noctor, G. (2007) A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during *Arabidopsis* rosette development. *Anal Biochem*, **363**, 58–69.

R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J. and Cuypers, A. (2008) Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta*, **227**, 1343–1349.

Remans, T., Opdenakker, K., Smeets, K., Mathijsen, D., Vangronsveld, J. and Cuypers, A. (2010) Metal-specific and NADPH oxidase dependent changes in lipoxygenase and NADPH oxidase gene expression in *Arabidopsis thaliana* exposed to cadmium or excess copper. *Funct Plant Biol*, **37**, 532–544.

Sandalio, L.M., Dalurzo, H.C., Gómez, M., Romero-Puertas, M.C. and del Río, L.A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J Exp Bot*, **52**, 2115–2126.

Schellingen, K., Van Der Straeten, D., Vandenbussche, F., Prinsen, E., Remans, T., Vangronsveld, J. and Cuypers, A. (2014) Cadmium-induced ethylene production and responses in *Arabidopsis thaliana* rely on *ACS2* and *ACS6* gene expression. *BMC Plant Biol*, **14**, 214.

Sharma, S.S. and Dietz, K.J. (2009) The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci*, **14**, 43–50.

Smeets, K., Ruytinx, J., Van Belleghem, F., Semane, B., Lin, D., Vangronsveld, J. and Cuypers, A. (2008) Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. *Plant Physiol Biochem*, **46**, 212–218.

Strodtkötter, I., Padmasree, K., Dinakar, C., Speth, B., Niazi, P.S., Wojtera, J., Voss, I., Do, P.T., Nunes-Nesi, A., Fernie, A.R., Linke, V., Raghavendra, A.S. and Scheibe, R. (2009) Induction of the AOX1D isoform of alternative oxidase in *A. thaliana* T-DNA insertion lines lacking isoform AOX1A is insufficient to optimize photosynthesis when treated with antimycin A. *Mol Plant*, **2**, 284–297.

Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J. and Millar, A.H. (2002) The impact of oxidative stress on *Arabidopsis* mitochondria. *Plant J*, **32**, 891–904.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, **3**, research0034.1–034.11.

Vanlerberghe, G.C., Cvetkovska, M. and Wang, J. (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? *Physiol Plant*, **137**, 392–406.

Vanlerberghe, G.C. (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci*, **14**, 6805–6847.

Wang, H., Liang, X., Huang, J., Zhang, D., Lu, H., Liu, Z. and Bi, Y. (2010) Involvement of ethylene and hydrogen peroxide in induction of alternative respiratory pathway in salt-treated *Arabidopsis* calluses. *Plant Cell Physiol*, **51**, 1754–1765.

Watanabe, C.K., Hachiya, T., Terashima, I. and Noguchi, K. (2008) The lack of alternative oxidase at low temperature leads to a disruption of the balance in carbon and nitrogen metabolism, and to an up-regulation of antioxidant defence systems in *Arabidopsis thaliana* leaves. *Plant Cell Environ*, **31**, 1190–1202.

Xu, F., Yuan, S., Zhang, D.W., Lv, X. and Lin, H.H. (2012) The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. *J Exp Bot*, **63**, 5705–5716.

SUPPLEMENTARY INFORMATION

Supplementary Table 5.1. *Forward (FW) and reverse (REV) primers used to determine gene expression levels via quantitative real-time PCR. E-E-jn, exon-exon junction; SAND, SAND family; TIP41-like, tonoplast intrinsic protein 41-like; UBC, ubiquitin-conjugating enzyme; AOX, alternative oxidase; ND, alternative NAD(P)H dehydrogenase; UCP, uncoupling protein; UPOX, upregulated by oxidative stress; TIR, Toll-Interleukin-1; LOX, lipoxygenase; RBOH, respiratory burst oxidase homologue; APX, ascorbate peroxidase; CAT, catalase; CSD, Cu/Zn superoxide dismutase; FSD, Fe superoxide dismutase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; MSD, Mn superoxide dismutase; Prx, peroxiredoxin; Trx, thioredoxin; ACS, ACC synthase; ACO, ACC oxidase; EBF, EIN3-binding F-box protein; ERF, ethylene response factor; ERS, ethylene response sensor; ETR, ethylene receptor. * ACS6 and ERS2 primer concentrations were increased to 900 nM. ** ERF1 primer concentrations were increased to 600 nM.*

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Reference genes					
AT2G28390	*SAND family*	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13 and 14	61	97.41%
AT4G34270	*TIP41-like*	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn and exon 2	61	90.60%
AT5G25760	*UBC*	FW: CTGCGACTCAGGGAATCTTCTAA REV: TTGTGCCATTGAATTGAACCC	E3-E4-jn and exon 4	61	102.61%
Genes encoding alternative oxidases (AOX)					
AT3G22370	*AOX1a*	FW: CTCTTCGTTGGCCTACCGATT REV: AACCATTCCAGGTACTGCTGCTAC	Exon 2 and 3	92	89.12%
AT1G32350	*AOX1d*	FW: ACCGTTCAAACTCTGAAAATACCG REV: GCAGCCACCGTCTCTAGCAA	Exon 1 and 2	86	85.07%
Genes encoding alternative NAD(P)H dehydrogenases (ND)					
AT1G07180	*NDA1*	FW: GCAAGGAAGGGAAAGGAATATCA REV: TTTCTCCAGCTGACGACTCGA	E7-E8-jn and exon 8	91	94.84%
AT2G29990	*NDA2*	FW: TAGTCGACCTTCGCGAGAGC REV: TCGAGTCAGGTACGCCGATC	Exon 7 and 8	92	91.95%
AT4G05020	*NDB2*	FW: GTCCAGAAGGTCCCATTAGGATG REV: CCCAGTGGTGCAAATTGTCC	Exon 8 and 10	202	87.60%
Genes encoding uncoupling proteins (UCP)					
AT5G58970	*UCP2*	FW: CGTTCCTTGAAACCTTCATTTGC REV: TGAAGTCTAACTTTGGCTGTGTCCA	Exon 1 and 2	91	101.47%
AT4G24570	*UCP4*	FW: TGTGCGGTGAAGACGGTTAAA REV: CAACAGTGAAAGGACCTTGCCT	Exon 1	91	102.47%

Supplementary Table 5.1. *Continued.*

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Genes encoding uncoupling proteins (UCP)					
AT2G22500	*UCP5*	FW: GACCCACCCGCTTGATCTAATC REV: AAAAGCAAGAGCTGGTCGGAG	Exon 1	91	100.29%
Genes encoding oxidative stress hallmark proteins					
AT2G21640	*UPOX*	FW: GACTTGTTTCAAAAACACCATGGAC REV: CACTTCCTTAGCCTCAATTTGCTTC	Exon 1 and 2	91	93.77%
AT2G43510	*Defensin-like*	FW: ATGGCAAAGGCTATCGTTTCC REV: CGTTACCTTGCGCTTCTATCTCC	Exon 1 and 2	91	98.42%
AT1G19020	*Unknown*	FW: GAAAATGGGACAAGGGTTAGACAAA REV: CCCAACGAAAACCAATAGCAGA	Exon 1	92	99.30%
AT1G05340	*Unknown*	FW: TCGGTAGCTCAGGGTAAAGTGG REV: CCAGGGCACAACAGCAACA	Exon 2 and 3	91	101.62%
AT1G57630	*TIR-class*	FW: ACTCAAACAGGCGATCAAAGGA REV: CACCAATTCGTCAAGACAACACC	Exon 1	91	94.56%
Genes encoding ROS producing enzymes					
AT1G55020	*LOX1*	FW: TTGGCTAAGGCTTTTGTCGG REV: GTGGCAATCACAAACGGTTC	Exon 6 and 7	101	99.13%
AT3G45140	*LOX2*	FW: TTTGCTCGCCAGACACTTG REV: GGGATCACCATAAACGGCC	Exon 3 and 4	102	86.65%
AT5G51060	*RBOHC*	FW: TCACCAGAGACTGGCACAATAAA REV: GATGCTCGACCTGAATGCTC	Exon 6 and 7	101	92.09%
AT5G47910	*RBOHD*	FW: AACTCTCCGCTGATTCCAACG REV: TGGTCAGCGAAGTCTTTAGATTCCT	Exon 1	91	99.24%
Genes encoding antioxidative enzymes					
AT1G07890	*APX1*	FW: TGCCACAAGGATAGGTCTGG REV: CCTTCCTTCTCTCCGCTCAA	Exon 5 and 6	101	94.43%
AT1G20630	*CAT1*	FW: AAGTGCTTCATCGGGAAGGA REV: CTTCAACAAAACGCTTCACGA	E5-E6-jn and exon 7	103	94.02%
AT4G35090	*CAT2*	FW: AACTCCTCCATGACCGTTGGA REV: TCCGTTCCCTGTCGAAATTG	Exon 2 and 3	76	96.32%
AT1G20620	*CAT3*	FW: TCTCCAACAACATCTCTTCCCTCA REV: GTGAAATTAGCAACCTTCTCGATCA	Exon 2 and 3	91	82.04%
AT1G08830	*CSD1*	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5 and E6-E7-jn	102	94.22%
AT2G28190	*CSD2*	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6 and E7-E8-jn	101	96.21%
AT4G25100	*FSD1*	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4 and E6-E7-jn	101	92.76%

Supplementary Table 5.1. *Continued.*

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Genes encoding antioxidative enzymes					
AT3G24170	*GR1*	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15 and 16	101	95.69%
Genes encoding mitochondrial antioxidative enzymes					
AT4G08390	*APX*	FW: GGACACCAGAGTGGCTGAAGTT REV: GCATCAGTGGGTAGGACAAGGAG	Exon 7 and 8	91	89.63%
AT1G19570	*DHAR*	FW: GATGGCTCTGGAAATCTGTGTGA REV: CGAGTGTGAGAAGAGCCCGT	Exon 1 and 2	92	94.77%
AT3G54660	*GR2*	FW: TAGGGTTGGAGAATGTTGGCG REV: GCCCAGATGGATGGAACAGAT	Exon 5 and 6	91	99.82%
AT3G10920	*MSD1*	FW: ATGTTTGGGAGCACGCCTAC REV: AACCTCGCTTGCATATTTCCA	Exon 5 and 6	101	87.33%
AT3G06050	*Prx*	FW: GCTATCAATGGTTGGGCAGAGA REV: GCCCCAAGCTTTTGTGAAATT	Exon 3 and 4	91	99.71%
AT2G35010	*Trx*	FW: AGGGCGGGATTTCGAACAC REV: ACCTCCCCTTTCTTCGAGCC	E4-E5-jn and exon 6	91	101.84%
Genes encoding ethylene-related enzymes					
AT1G01480	*ACS2*	FW: CATGTTCTGCCTTGCGGATC REV: ACCTGTCCGCCACCTCAAGT	Exon 3 and 4	91	104.41%
AT4G11280	*ACS6**	FW: TTAGCTAATCCCGGCGATGG REV: ACAAGATTCACTCCGGTTCTCCA	Exon 3 and 4	92	97.88%
AT1G62380	*ACO2*	FW: TCTACGTTCGTCACCTCCCTCA REV: CTCTTACCAAAGTCTTTCATGGCC	Exon 2 and 3	91	100.76%
AT5G25350	*EBF2*	FW: TGGAACTTGCCTGCTGTTAGTG REV: CAGGACACCGTGAAAGGTCAA	Exon 1	91	93.73%
AT3G23240	*ERF1***	FW: TCCTCGGCGATTCTCAATTTT REV: CAACCGGAGAACAACCATCCT	Exon 1	91	98.10%
AT2G40940	*ERS1*	FW: TAGAAAACGTGGCGGATCAGG REV: TGCTCCATAAGCTGGTCACGA	Exon 1 and 2	92	93.61%
AT1G04310	*ERS2**	FW: AGTCTCAACGCTTGCCAAAACAT REV: CAACTGAGACGCTTTTCACCAAAC	Exon 1 and 2	93	94.90%
AT3G23150	*ETR2*	FW: TTCGAACCGGGCAGTTACAC REV: AATGGCGGTAAGGCAATCG	Exon 2	91	87.50%

Supplementary Table 5.2. *Quantitative real-time PCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin et al. (2009). * All procedures were performed according to the manufacturer's protocols.*

Sample/Template	
Source	Leaves (entire rosette) of *Arabidopsis thaliana* plants cultivated in hydroponics
Method of preservation	Liquid nitrogen
Storage time	3 weeks at -70 °C
Handling	Frozen
Extraction method	Phenol-free Total RNA isolation: RNAqueous® Total RNA Isolation Kit* (Ambion, Life Technologies, Belgium)
RNA: DNA-free	TURBO DNA-*free*™ Kit* (Ambion, Life Technologies, Belgium) Design of intron-spanning primers whenever possible
Concentration	NanoDrop®: ND-1000 Spectrophotometer (ThermoScientific, USA)
Assay optimisation and validation	
Accession number	Supplementary Table 5.1
Amplicon details	Exon location and amplicon size: Supplementary Table 5.1
Primer sequences	Supplementary Table 5.1
In silico	Primers were blasted using the BLAST tool at http://arabidopsis.org/
Empirical	A primer concentration of 300 nM was used unless stated otherwise Annealing temperature: 60 °C
Priming conditions	Combination of oligodT-primers and random hexamers
PCR efficiency	Dilution series (slope, y-intercept and r^2; Supplementary Table 5.1)
Linear dynamic range	Samples are situated within the range of the efficiency curve
Reverse transcription - PCR	
Protocols	As stated in the Materials and Methods section
Reagents	As stated in the Materials and Methods section
No template control (NTC)	Cq and dissociation curve verification
Data analysis	
Specialist software	7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) Software v2.0.1
Statistical justification	4 biological replicates Outliers were eliminated after statistical validation using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05 Log transformation of the data Two-way ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparisons using R version 2.13.1
Normalisation	3 reference genes were selected as described in the Materials and Methods section

Supplementary Table 5.3. *Relative leaf transcript levels of genes encoding components of the mitochondrial alternative respiratory pathway, oxidative stress hallmark proteins, ROS producing, antioxidative and ethylene-related enzymes in Arabidopsis thaliana. Transcript levels were measured using quantitative real-time PCR in leaf samples of 19-days-old wild-type (WT) and aox1a knockout (aox1a) plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading: p < 0.05; p < 0.01 and p < 0.05; p < 0.01 for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks (p < 0.05) (two-way ANOVA per time point). Abbreviations: Supplementary Table 5.1.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding alternative oxidases (AOX)					
	Control	1.00 ± 0.07	ND	1.00 ± 0.06	ND
AOX1a	**5 µM Cd**	13.94 ± 0.49	ND	3.06 ± 0.45	ND
	10 µM Cd	14.97 ± 0.90	ND	3.38 ± 0.30	ND
	Control	1.00 ± 0.05	1.00 ± 0.32	1.00 ± 0.53	1.00 ± 0.18
AOX1d	**5 µM Cd**	4196.03 ± 970.82	7277.37 ± 1662.32	669.00 ± 353.28	21492.72 ± 3594.85*
	10 µM Cd	6872.25 ± 231.20	15754.05 ± 2025.77	1100.64 ± 281.55	7947.95 ± 2685.70*
Genes encoding alternative NAD(P)H dehydrogenases (ND)					
	Control	1.00 ± 0.00	1.00 ± 0.01	1.00 ± 0.04	1.00 ± 0.01
NDA1	**5 µM Cd**	0.37 ± 0.09	0.46 ± 0.09	0.50 ± 0.02	0.58 ± 0.08
	10 µM Cd	0.28 ± 0.01	0.29 ± 0.04	0.49 ± 0.02	0.46 ± 0.02
	Control	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.04
NDA2	**5 µM Cd**	27.68 ± 0.31	21.81 ± 3.98	6.56 ± 2.08	9.39 ± 1.13
	10 µM Cd	26.40 ± 1.53	26.27 ± 2.32	5.62 ± 0.13	6.62 ± 0.67
	Control	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.07	1.00 ± 0.10
NDB2	**5 µM Cd**	14.26 ± 2.36	11.55 ± 2.01	3.78 ± 1.03	4.86 ± 0.02
	10 µM Cd	14.71 ± 0.92	12.69 ± 0.32	3.08 ± 0.20	3.24 ± 0.19

Supplementary Table 5.3. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding uncoupling proteins (UCP)					
UCP2	**Control**	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.05
	5 µM Cd	0.52 ± 0.06	0.60 ± 0.06	0.88 ± 0.04	0.91 ± 0.06
	10 µM Cd	0.47 ± 0.01	0.41 ± 0.02	0.93 ± 0.04	0.94 ± 0.04
UCP4	**Control**	1.00 ± 0.16	1.00 ± 0.19	1.00 ± 0.04	1.00 ± 0.13
	5 µM Cd	17.26 ± 1.71	17.27 ± 0.83	7.34 ± 1.63	8.18 ± 1.00
	10 µM Cd	9.16 ± 0.04	11.44 ± 0.91	2.81 ± 0.02	3.85 ± 0.75
UCP5	**Control**	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.09
	5 µM Cd	12.87 ± 1.88	12.87 ± 2.12	4.11 ± 0.60	7.25 ± 1.25*
	10 µM Cd	9.55 ± 0.95	8.78 ± 0.93	2.63 ± 0.13	3.46 ± 0.30
Genes encoding oxidative stress hallmark proteins					
UPOX	**Control**	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.07	1.00 ± 0.05
	5 µM Cd	5.39 ± 1.03	4.26 ± 0.35	4.00 ± 0.85	15.91 ± 3.88*
	10 µM Cd	11.44 ± 0.14	10.94 ± 1.19	4.42 ± 0.47	6.31 ± 0.50
Defensin-like	**Control**	1.00 ± 0.08	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.17
	5 µM Cd	38.48 ± 6.90	27.02 ± 4.80	51.69 ± 14.76	74.08 ± 15.78
	10 µM Cd	124.68 ± 9.22	77.13 ± 14.51	174.30 ± 15.91	260.32 ± 26.12
Unknown (AT1G19020)	**Control**	1.00 ± 0.15	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.20
	5 µM Cd	200.21 ± 35.71	165.48 ± 26.55	25.73 ± 5.33	180.47 ± 22.67*
	10 µM Cd	205.81 ± 14.27	209.33 ± 25.72	17.27 ± 2.05	32.30 ± 8.15
Unknown (AT1G05340)	**Control**	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.12	1.00 ± 0.06
	5 µM Cd	97.82 ± 25.90	59.14 ± 12.52	55.65 ± 31.69	114.38 ± 16.70
	10 µM Cd	151.59 ± 13.25	130.16 ± 23.93	43.36 ± 13.04	87.56 ± 23.77
TIR-class	**Control**	1.00 ± 0.28	1.00 ± 0.19	1.00 ± 0.27	1.00 ± 0.28
	5 µM Cd	334.85 ± 7.28	281.33 ± 49.96	21.41 ± 4.26	104.07 ± 10.09*
	10 µM Cd	324.78 ± 34.28	315.92 ± 30.68	18.29 ± 1.37	27.87 ± 5.38

Supplementary Table 5.3. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding ROS producing enzymes					
	Control	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.09
LOX1	**5 µM Cd**	2.74 ± 0.43	2.34 ± 0.29	1.85 ± 0.49	2.22 ± 0.38
	10 µM Cd	7.14 ± 0.52	6.53 ± 0.97	4.71 ± 0.47	6.72 ± 1.21
	Control	1.00 ± 0.09	1.00 ± 0.01	1.00 ± 0.12	1.00 ± 0.23
LOX2	**5 µM Cd**	6.20 ± 0.62	7.51 ± 0.31	3.92 ± 0.92	3.62 ± 0.29
	10 µM Cd	6.55 ± 0.77	5.92 ± 0.09	7.20 ± 0.70	8.24 ± 0.64
	Control	1.00 ± 0.39	1.00 ± 0.20	1.00 ± 0.43	1.00 ± 0.20
RBOHC	**5 µM Cd**	675.16 ± 167.35	269.82 ± 48.76	18.72 ± 10.98	665.24 ± 181.52*
	10 µM Cd	780.51 ± 80.67	462.06 ± 82.09	11.38 ± 4.54	74.90 ± 19.83
	Control	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.04
RBOHD	**5 µM Cd**	3.40 ± 0.38	3.81 ± 0.18	2.58 ± 0.52	3.59 ± 0.46
	10 µM Cd	3.75 ± 0.31	3.53 ± 0.23	2.81 ± 0.08	3.21 ± 0.21
Genes encoding antioxidative enzymes					
	Control	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.02
APX1	**5 µM Cd**	1.52 ± 0.10	1.31 ± 0.02	1.16 ± 0.12	1.44 ± 0.07
	10 µM Cd	1.89 ± 0.12	1.65 ± 0.12	1.31 ± 0.06	1.29 ± 0.06
	Control	1.00 ± 0.04	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.02
CAT1	**5 µM Cd**	2.41 ± 0.21	2.16 ± 0.18	1.34 ± 0.12	2.16 ± 0.59
	10 µM Cd	3.88 ± 0.08	2.98 ± 0.25	2.85 ± 0.54	4.30 ± 0.79
	Control	1.00 ± 0.00	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.03
CAT2	**5 µM Cd**	0.16 ± 0.02	0.37 ± 0.07*	0.40 ± 0.03	0.43 ± 0.08
	10 µM Cd	0.15 ± 0.01	0.16 ± 0.04	0.41 ± 0.02	0.36 ± 0.02
	Control	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.03
CAT3	**5 µM Cd**	1.49 ± 0.24	1.62 ± 0.17	2.80 ± 0.27	3.51 ± 0.15
	10 µM Cd	1.85 ± 0.21	1.85 ± 0.09	4.19 ± 0.40	4.26 ± 0.18

Supplementary Table 5.3. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding antioxidative enzymes					
	Control	1.00 ± 0.00	1.00 ± 0.03	1.00 ± 0.12	1.00 ± 0.02
CSD1	**5 µM Cd**	1.92 ± 0.32	1.35 ± 0.17	0.62 ± 0.05	0.66 ± 0.05
	10 µM Cd	2.28 ± 0.26	2.15 ± 0.36	0.25 ± 0.03	0.25 ± 0.04
	Control	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.15	1.00 ± 0.04
CSD2	**5 µM Cd**	0.47 ± 0.02	0.38 ± 0.03	0.13 ± 0.02	0.13 ± 0.01
	10 µM Cd	0.38 ± 0.07	0.40 ± 0.07	0.04 ± 0.00	0.03 ± 0.00
	Control	1.00 ± 0.22	1.00 ± 0.22	1.00 ± 0.55	1.00 ± 0.36
FSD1	**5 µM Cd**	1.88 ± 0.86	5.10 ± 1.19	1.27 ± 0.37	7.61 ± 1.49
	10 µM Cd	1.48 ± 0.38	1.45 ± 0.53	2.35 ± 0.34	9.67 ± 0.57
	Control	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.05
GR1	**5 µM Cd**	2.54 ± 0.30	2.47 ± 0.20	1.55 ± 0.16	1.51 ± 0.18
	10 µM Cd	3.58 ± 0.14	2.92 ± 0.08	1.74 ± 0.05	2.19 ± 0.17
Genes encoding mitochondrial antioxidative enzymes					
	Control	1.00 ± 0.03	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.03
APX	**5 µM Cd**	0.92 ± 0.04	1.03 ± 0.06	0.55 ± 0.10	0.78 ± 0.08
	10 µM Cd	0.80 ± 0.09	0.76 ± 0.03	0.82 ± 0.08	0.89 ± 0.02
	Control	1.00 ± 0.10	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.10
DHAR	**5 µM Cd**	1.71 ± 0.22	1.68 ± 0.10	3.13 ± 0.65	2.87 ± 0.23
	10 µM Cd	1.79 ± 0.14	1.62 ± 0.27	5.86 ± 0.20	6.19 ± 0.05
	Control	1.00 ± 0.02	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.01
GR2	**5 µM Cd**	0.51 ± 0.08	0.58 ± 0.08	0.77 ± 0.05	0.78 ± 0.07
	10 µM Cd	0.44 ± 0.02	0.40 ± 0.02	0.90 ± 0.02	0.99 ± 0.06
	Control	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.04
MSD1	**5 µM Cd**	0.96 ± 0.01	1.07 ± 0.05	1.01 ± 0.02	1.10 ± 0.02
	10 µM Cd	1.19 ± 0.09	1.06 ± 0.04	1.23 ± 0.03	1.35 ± 0.08
	Control	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.02	1.00 ± 0.04
Prx	**5 µM Cd**	1.34 ± 0.05	1.36 ± 0.08	1.69 ± 0.04	1.58 ± 0.04
	10 µM Cd	1.42 ± 0.04	1.38 ± 0.07	2.04 ± 0.09	2.16 ± 0.06

Supplementary Table 5.3. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding mitochondrial antioxidative enzymes					
	Control	1.00 ± 0.07	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.04
Trx	**5 µM Cd**	1.19 ± 0.07	1.14 ± 0.01	1.26 ± 0.01	1.30 ± 0.03
	10 µM Cd	1.07 ± 0.05	1.00 ± 0.06	1.55 ± 0.02	1.51 ± 0.10
Genes encoding ethylene-related enzymes					
	Control	1.00 ± 0.14	1.00 ± 0.28	1.00 ± 0.21	1.00 ± 0.02
ACS2	**5 µM Cd**	2040.15 ± 476.87	2452.25 ± 580.41	402.52 ± 208.50	605.31 ± 144.54
	10 µM Cd	4556.74 ± 390.65	6211.56 ± 1282.68	949.22 ± 389.95	1248.24 ± 393.35
	Control	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.08
ACS6	**5 µM Cd**	26.99 ± 0.52	20.53 ± 2.73	4.01 ± 0.55	10.11 ± 1.24*
	10 µM Cd	24.66 ± 3.31	20.89 ± 1.82	3.42 ± 0.32	4.56 ± 1.01
	Control	1.00 ± 0.02	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.01
ACO2	**5 µM Cd**	4.45 ± 0.91	2.63 ± 0.23	1.81 ± 0.34	2.76 ± 0.37
	10 µM Cd	8.39 ± 0.86	6.34 ± 0.55	3.57 ± 0.46	4.34 ± 0.70
	Control	1.00 ± 0.04	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.03
EBF2	**5 µM Cd**	4.46 ± 0.52	3.80 ± 0.68	2.09 ± 0.21	3.07 ± 0.31*
	10 µM Cd	6.70 ± 0.14	5.54 ± 0.12	3.69 ± 0.07	3.87 ± 0.29
	Control	1.00 ± 0.16	1.00 ± 0.22	1.00 ± 0.22	1.00 ± 0.19
ERF1	**5 µM Cd**	538.80 ± 69.92	753.97 ± 93.66	42.85 ± 14.95	90.53 ± 20.61
	10 µM Cd	767.39 ± 29.69	825.11 ± 29.28	66.32 ± 3.25	124.71 ± 19.43
	Control	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.01
ERS1	**5 µM Cd**	2.92 ± 0.06	3.35 ± 0.36	2.10 ± 0.09	2.53 ± 0.16
	10 µM Cd	3.22 ± 0.06	3.11 ± 0.14	2.34 ± 0.01	2.85 ± 0.15
	Control	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.04
ERS2	**5 µM Cd**	2.60 ± 0.22	2.76 ± 0.41	1.17 ± 0.10	1.54 ± 0.16
	10 µM Cd	3.28 ± 0.31	2.84 ± 0.17	1.57 ± 0.04	1.91 ± 0.10
	Control	1.00 ± 0.17	1.00 ± 0.08	1.00 ± 0.19	1.00 ± 0.07
ETR2	**5 µM Cd**	3.54 ± 0.52	3.87 ± 0.49	3.00 ± 0.45	6.07 ± 0.12*
	10 µM Cd	5.05 ± 0.29	5.07 ± 0.59	5.54 ± 0.46	9.13 ± 0.59*

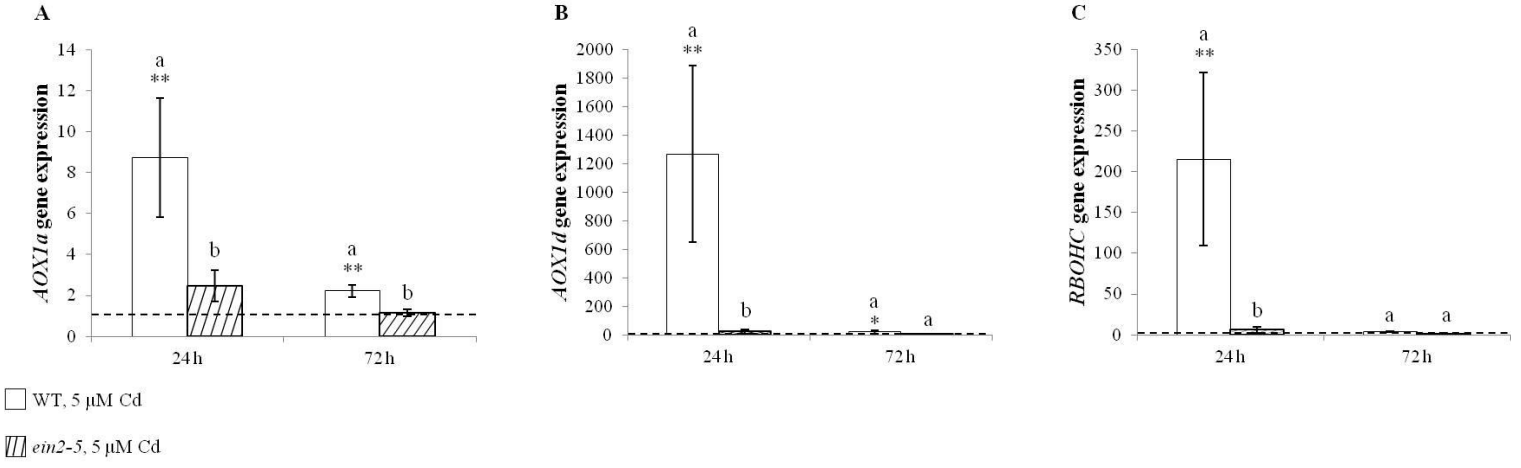
Supplementary Table 5.4. *Overall genotype * treatment interaction effects represented by the p-values for all measured genes in wild-type and aox1a knockout Arabidopsis thaliana leaves per time point. Significant interaction effects are indicated in bold.*

	24 h	72 h
AOX1a	**0.000**	**0.000**
AOX1d	0.177	**0.001**
NDA1	0.563	0.375
NDA2	0.313	0.260
NDB2	0.724	0.345
UCP2	0.122	0.911
UCP4	0.593	0.655
UCP5	0.928	**0.043**
UPOX	0.796	**0.001**
Defensin-like	0.269	0.537
Unknown (AT1G19020)	0.782	**0.000**
Unknown (AT1G05340)	0.690	0.208
TIR-class	0.697	**0.005**
LOX1	0.851	0.625
LOX2	0.258	0.825
RBOHC	0.224	**0.010**
RBOHD	0.411	0.223
APX1	0.510	0.087
CAT1	0.222	0.399
CAT2	**0.018**	0.605
CAT3	0.860	0.257
CSD1	0.449	0.943
CSD2	0.430	0.580
FSD1	0.123	0.522
GR1	0.285	0.285
APX	0.546	0.163
DHAR	0.781	0.959
GR2	0.425	0.755
MSD1	0.141	0.375
Prx	0.908	0.174
Trx	0.771	0.753
ACS2	0.771	0.688
ACS6	0.356	**0.013**
ACO2	0.196	0.336
EBF2	0.500	**0.036**
ERF1	0.471	0.195
ERS1	0.409	0.110
ERS2	0.579	0.252
ETR2	0.901	**0.020**

Supplementary Table 5.5. *Relative leaf transcript levels of genes encoding alternative oxidases (AOX1a and AOX1d) and respiratory burst oxidase homologue c (RBOHC) in different genotypes of Arabidopsis thaliana. Transcript levels were measured using quantitative real-time PCR in leaf samples of 19-days-old wild-type (WT) and acs2-1/acs6-1 double knockout (acs2-1/6-1), ethylene-insensitive ein2-1 or ein2-5 mutants grown under control conditions. Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed WT after 24 h set at 1.00. Asterisks indicate significant differences as compared to the WT (p < 0.05) (two-way ANOVA).*

	24 h			**72 h**		
	acs2-1/6-1	***ein2-1***	***ein2-5***	***acs2-1/6-1***	***ein2-1***	***ein2-5***
AOX1a	1.05 ± 0.08	1.92 ± 0.27*	2.13 ± 0.37*	2.00 ± 0.40	1.69 ± 0.24	1.76 ± 0.27
AOX1d	0.68 ± 0.38	90.52 ± 24.32*	20.12 ± 10.80	11.49 ± 9.01	40.59 ± 18.28	13.44 ± 2.71
RBOHC	1.33 ± 0.48	4.61 ± 0.87*	10.60 ± 7.08	6.04 ± 4.51	1.54 ± 0.56	1.44 ± 0.52

Supplementary Figure 5.1.



Supplementary Figure 5.1. *Relative leaf transcript levels of alternative oxidases AOX1a (A), AOX1d (B) and respiratory burst oxidase homologue C (C) in Arabidopsis thaliana. Transcript levels were measured via quantitative real-time PCR in leaf samples of 19-days-old wild-type (WT) plants and ethylene-insensitive ein2-5 mutants exposed to 5 µM $CdSO_4$ (WT: white; mutant: white striped) during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks: * $p < 0.05$, ** $p < 0.01$. Different letters denote significant differences between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA).*

CHAPTER 6

Metabolic responses of *Arabidopsis thaliana* roots and leaves to sublethal Cd exposure are differentially influenced by ALTERNATIVE OXIDASE1a

Els Keunen, Igor Florez-Sarasa, Toshihiro Obata, Marijke Jozefczak, Tony Remans, Jaco Vangronsveld, Alisdair R. Fernie, Ann Cuypers (2014) Metabolic responses of *Arabidopsis thaliana* roots and leaves to sublethal Cd exposure are differentially influenced by ALTERNATIVE OXIDASE1a. In preparation.

ABSTRACT

In this study, metabolic responses in roots and leaves of intact *Arabidopsis thaliana* plants to sublethal cadmium (Cd) exposure were characterised over time. In Cd-exposed roots, the initial drop in sugar and organic acid levels is indicative of an increased TCA cycle activity to support stress defence mechanisms. On the other hand, sugars and organic acids accumulated in leaves of Cd-exposed plants. Although this could be related to starch degradation as backup energy store during stress, root-to-shoot signalling mechanisms might also be involved in this opposite response between both tissues. These results also point towards a differential involvement of mitochondria in roots and leaves during Cd exposure. The mitochondrial alternative oxidase (AOX) is commonly suggested to modulate metabolic homeostasis and is induced at transcript and protein level in Cd-exposed *A. thaliana* plants. Therefore, metabolic responses of wild-type plants were compared to those in *aox1a* knockout mutants. Whereas a lack of AOX1a function does not impact primary metabolism under control conditions, differential responses were noted in both roots and leaves under Cd exposure. Our results confirm the previously suggested link between ethylene and AOX in the leaves. Furthermore, an increased respiratory flux dependent on amino acid degradation is suggested in Cd-exposed leaves and might require AOX1a. Finally, our results suggest a reciprocal relation between AOX1a and antioxidative defence mechanisms, in particular glutathione (GSH), at different biological levels in roots of *A. thaliana* plants exposed to Cd.

Keywords:

alternative oxidase, *Arabidopsis thaliana*, cadmium, metabolic profiling, oxidative challenge.

6.1. INTRODUCTION

Soil pollution with the toxic metal cadmium (Cd) is challenging for plants as it is readily taken up by their roots and transported to the above-ground tissues. Subsequently, Cd exposure affects photosynthesis, respiration and the uptake of nutrients and water, thereby significantly reducing plant growth and productivity. It is of utmost importance to uncover Cd phytotoxicity mechanisms and potentially improve our approaches to deal with polluted soils (DalCorso *et al*. 2008, Vangronsveld *et al*. 2009, Seth *et al*. 2012).

Plant responses to Cd have been extensively studied using transcriptomics (Herbette *et al*. 2006) and proteomics (Sarry *et al*. 2006, Dupae *et al*. 2014) in the model organism *Arabidopsis thaliana*. However, less is known about its impact at the metabolic level. Sarry *et al*. (2006) explored metabolic responses of *A. thaliana* cells exposed to $CdCl_2$ concentrations ranging from 0.5 to 200 µM within 24 h. A pivotal role was attributed to phytochelatins (PCs), as their levels increased with the Cd concentration applied. In agreement with these results, PC accumulation was demonstrated in roots and leaves of Cd-exposed *A. thaliana* plants (Jozefczak *et al*. 2014). Another recent study by Sun *et al*. (2010) used metabolite profiling to characterise Cd stress responses in *A. thaliana* plants exposed to 5 or 50 µM $CdCl_2$ during 2 weeks. Although root and leaf responses were not discriminated, the study highlighted the importance of antioxidative defence in plant resistance to Cd stress. Indeed, Cd phytotoxicity is strongly related to the generation of reactive oxygen species (ROS) imposing a cellular oxidative challenge (Cuypers *et al*. 2012 and references therein).

At the subcellular level, the mitochondrial electron transport chain (ETC) is one of the primary ROS production sites during Cd exposure (Heyno *et al*. 2008, Bi *et al*. 2009). In the plant mitochondrial ETC, two terminal oxidases are present. While cytochrome c oxidase (complex IV) reduces O_2 and concurrently pumps protons across the inner membrane, the alternative oxidase (AOX) reduces O_2 without proton translocation. Furthermore, its activity dampens mitochondrial ROS production under physiological conditions (Cvetkovska and Vanlerberghe 2012), which might imply a role for this enzyme during the Cd-induced oxidative challenge. Recently, Vanlerberghe (2013) reviewed the metabolic importance of AOX as it allows plants to flexibly alter the degree of coupling between carbon metabolism, ETC activity and ATP turnover. As AOX is

activated at transcript and protein level in Cd-exposed *A. thaliana* plants (Keunen *et al*. 2013a), its activity might influence Cd stress responses by modulating redox and metabolic homeostasis.

The goal of the current study is twofold as it aims to unravel (1) how exposure to environmentally realistic Cd concentrations affects primary plant metabolism over time in roots and leaves of *A. thaliana* plants and (2) if and how AOX modulates this metabolic response. To this end, we characterised the metabolic responses of wild-type (WT) and *aox1a* knockout plants exposed to sublethal Cd concentrations (Keunen *et al*. 2011) during 24 and 72 h (Keunen *et al*. 2013a) using gas chromatography-mass spectrometry (GC-MS). Additional parameters (gene expression, enzyme capacity) were included to support the results obtained at the metabolic level.

6.2. MATERIALS AND METHODS

6.2.1. Plant culture and cadmium exposure

Wild-type (WT), *aox1a* knockout (SALK_084897 T-DNA insertional line for *AOX1a*, Alonso *et al*. 2003, Watanabe *et al*. 2008) and glutathione (GSH)-deficient *cad2-1* (Cobbett *et al*. 1998), *pad2-1* (Parisy *et al*. 2006) and *rax1-1* (Ball *et al*. 2004) genotypes of the Columbia ecotype were confirmed using PCR as described in the above-mentioned papers. All seeds were surface-sterilised and grown on hydroponics (Smeets *et al*. 2008a), except that purified sand was used instead of rock wool (Keunen *et al*. 2011). A modified Hoagland nutrient solution was used (section 3.2.1., Smeets *et al*. 2008a) and growth conditions were set at a 12 h photoperiod, 65% relative humidity and day/night temperatures of 22 °C and 18 °C respectively. Light was provided by Philips Green-Power LED modules. Using a combination of blue, red and far-red modules, a spectrum simulating the photosynthetic active radiation (PAR) of sunlight was obtained. The PAR at the rosette level was 170 µmol m^{-2} s^{-1}. After 19 days of growth, control samples (0 h) were taken prior to exposure of the plants to 5 or 10 µM $CdSO_4$ supplied to the roots or further growth under control conditions. After 24 and 72 h, root and leaf (entire rosette) samples were taken and the fresh weight was determined. Samples were snap frozen in liquid nitrogen and stored at -70 °C for further analyses.

6.2.2. Metabolic profiling

Metabolite extractions, derivatisation and gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analyses were executed as described before (Lisec *et al*. 2006). Frozen root and leaf samples were disrupted in 2 mL microcentrifuge tubes using two stainless steel beads and the Retsch Mixer Mill MM 400 (Retsch, Belgium) under frozen conditions. Sample powders (50 mg) were mixed with 700 µL methanol, and 30 µL ribitol (stock solution 0.2 mg ml^{-1}) was added as an internal standard. After incubation (70 °C, 15 min), the extract was centrifuged (10 min, 11 000 *g*). To separate polar and non-polar metabolites, the supernatant was mixed with 375 µL chloroform and 750 µL water and centrifuged (15 min, 2 200 *g*). For further analysis, 150 µL of the upper polar phase was dried *in vacuo* and stored at -70 °C.

The pellet was resuspended in 40 µL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) and derivatised for 2 h at 37 °C. After addition of 70 µL *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) containing 20 µL mL^{-1} retention time standard mixture of fatty acid methylesters (FAMEs), the mixture was incubated at 37 °C during 30 min (Lisec *et al*. 2006).

The GC-TOF-MS system consisted of a CTC CombiPAL autosampler (CTC Analytics, Switzerland), an Agilent 6890N gas chromatograph (Agilent Technologies, USA) and a LECO Pegasus III time-of-flight mass spectrometer running in EI^+ mode (Leco Instruments, USA). A volume of 1 µL of each sample was injected at 230 °C with helium carrier gas flow set at 2 ml min^{-1}. Chromatography was performed with a 30-m MDN-35 capillary column (Macherey-Nagel, Germany). The initial temperature was set at 80 °C, followed by a 15 °C min^{-1} ramp to 330 °C held during 6 min. The transfer line and ion source were set at a temperature of 250 °C. After a solvent delay of 170 s, mass spectra were recorded at 20 scans per s within a mass to charge ratio range between 70 and 600 (Lisec *et al*. 2006). Metabolites were identified by comparison with database entries of authentic standards (Kopka *et al*. 2005, Schauer *et al*. 2005). Per condition (tissue, genotype, Cd exposure and time point), values are presented as the mean ± S.E. of 6 biological replicates.

6.2.3. Gene expression analysis

Frozen root samples were disrupted as described before (section 6.2.2.). From the disrupted tissues, RNA was extracted using the RNAqueous® Total RNA Isolation Kit (Ambion, Life Technologies, Belgium). Genomic DNA was removed via the TURBO DNA-*free*™ Kit (Ambion) and reverse transcription was carried out using the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa Bio Inc., Westburg, the Netherlands). A tenfold dilution of the cDNA was made in 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM Na_2-EDTA, pH 8.0) and stored at -20 °C. Quantitative real-time PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) and the Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions and as described before (Chapter 5). Gene-specific forward and reverse primers (300 nM, Supplementary Table 6.1) were designed and optimised via the Primer Express software (v2.0, Applied Biosystems).

Gene expression levels were calculated via the $2^{-\Delta Cq}$ method relative to the sample with the highest expression (minimum Cq). All data were normalised to the geometric average of the $2^{-\Delta Cq}$ values of stable reference genes (Remans *et al*. 2008) selected by geNorm (v3.5, Vandesompele *et al*. 2002) and Normfinder (v0.953, Andersen *et al*. 2004) algorithms (Supplementary Table 6.2). All details of our workflow according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines as described by Bustin *et al*. (2009) are shown in Supplementary Table 6.3.

6.2.4. Analysis of enzyme capacities

Frozen root (150 mg) samples were crushed in liquid nitrogen using a cooled mortar and pestle. Samples were transferred to a 15 mL Falcon tube on ice and agitated for 30 min at 4 °C after addition of 2 mL ice-cold extraction buffer (0.1 M Tris-HCl, 5 mM EDTA, 1% polyvinylpyrrolidone (PVP) K30, 5 mM dithioerythritol (DTE) and 1% Nonidet P-40, pH 7.8). After centrifugation (30 min, 50 000 *g*, 4 °C), samples were precipitated using ammonium sulfate ($(NH_4)_2SO_4$, 40%) during 30 min at 4 °C while shaking. Samples were centrifuged (30 min, 50 000 *g*, 4 °C) and subjected to a second precipitation step ($(NH_4)_2SO_4$, 80%), shaken and centrifuged as described above. Pellets were resuspended in 1.75 mL Tris-HCl buffer (25 mM, pH 7.8) and protein extracts

were desalted using PD-10 Desalting columns (GE Healthcare, Belgium). After centrifugation (2 min, 950 *g*, 4 °C), eluates were snap-frozen in liquid nitrogen and stored at -70 °C before enzyme capacities were spectrophotometrically measured at 25 °C under non-limiting reaction conditions using the UV-1600 spectrophotometer (Shimadzu, Germany).

Superoxide dismutase (SOD, EC 1.15.1.1) capacity was assessed at 550 nm by monitoring the inhibition of cytochrome c-mediated superoxide ($O_2^{\bullet-}$) oxidation in the samples relative to a blank (McCord and Fridovich 1969). The removal of H_2O_2 by the action of catalase (CAT, EC 1.11.1.6) was monitored at 240 nm according to Bergmeyer *et al*. (1974). Using the chemical peroxidase (POD) substrates guaiacol or syringaldazine, the capacity of guaiacol peroxidase (GPOD) and syringaldazine peroxidase (SPOD) (EC 1.11.1.7) was analysed at 436 and 530 nm as described by Bergmeyer *et al*. (1974) and Imberty *et al*. (1984) respectively.

Capacities of all NAD(P)H-dependent enzymes [glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), glutamate dehydrogenase (GDH, EC 1.4.1.2), glutathione reductase (GR, EC 1.6.4.2), isocitrate dehydrogenase (ICDH, EC 1.1.1.42) and NADP-malic enzyme (ME, EC 1.1.1.40)] were monitored at 340 nm according to Bergmeyer *et al*. (1974). Except for SOD, all enzyme capacities were calculated based on the Beer-Lambert law, taking into account specific extinction coefficients (ε_{CAT} = 40 M^{-1} cm^{-1}; ε_{SPOD} = 11.6 M^{-1} cm^{-1}; ε_{GPOD} = 25.5 M^{-1} cm^{-1}; $\varepsilon_{NAD(P)H\text{-dependent enzymes}}$ = 6.22 M^{-1} cm^{-1}).

6.2.5. Statistical analyses

All statistical analyses were performed using R version 2.13.1 (R Development Core Team 2011). The statistical analysis used is indicated in the caption of each Table or Figure. Datasets were statistically analysed with ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparisons. Both normality and homoscedasticity were checked; transformations were applied when necessary to approximate normality. If normality could not be reached, a non-parametric Kruskal-Wallis test, followed by the Wilcoxon rank sum test was used. Outliers were determined using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05. For gene expression data,

normalised relative quantities were log transformed prior to statistical analysis (Supplementary Table 6.3).

6.3. RESULTS

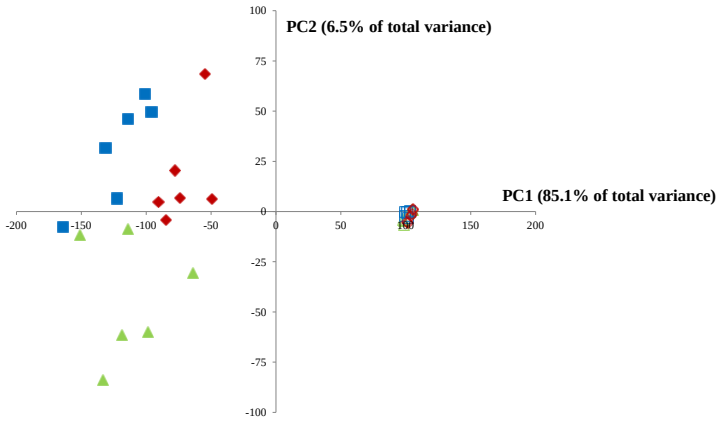
From previous research, it is clear that *A. thaliana* roots and leaves differentially react to sublethal Cd exposure in a hydroponic system when comparing responses at transcript, protein and enzyme capacity level (Smeets *et al*. 2008b, Cuypers *et al*. 2011, Keunen *et al*. 2013a, Jozefczak *et al*. 2014). Therefore, metabolic responses were first studied in WT roots and leaves, both under control and 5 or 10 µM Cd conditions. Two time points (24 and 72 h after exposure) were selected based on prior kinetic studies in Cd-exposed WT plants (Keunen *et al*. 2013a, Jozefczak *et al*. 2014).

6.3.1. Time-course root versus leaf primary metabolism in unexposed wild-type plants

A total of 35 (in roots) and 34 (in leaves) polar metabolites were identified in control plants using GC-TOF-MS and were classified as amino acids (proteinogenic or non-proteinogenic), organic acids, sugars or others (Supplementary Table 6.4). To obtain an overall picture of the differences in metabolite abundance during the entire experimental time course (0, 24 and 72 h) in both roots and leaves under control conditions, metabolite profiles were subjected to principal component analysis (PCA, Figure 6.1). Samples from both tissues were clearly separated, with a higher degree of variance in leaves as compared to roots (Figure 6.1A). Within roots, samples taken at the different time points did not show distinct clustering patterns. On the other hand, leaf samples taken after 24 and 72 h generally clustered separately from those at 0 h (Figure 6.1A). When performing the same PCA on a subset of metabolites, it was clear that time-course changes were mostly pronounced when comparing sugar levels in both tissues (Figure 6.1B). While variance was still limited in the roots, leaf control samples taken at 0 h clearly clustered away from those after 24 and 72 h (Figure 6.1B). When performing PCA on all commonly detected metabolites in both organs, principal component 1 (PC1) accounted for 85.1% and principal component 2 (PC2) for 6.5% of the total variance within the dataset (Figure 6.1A). Similar results were obtained when analysing sugars only,

with PC1 and PC2 accounting for 86.4% and 13.5% of total variance respectively (Figure 6.1B).

A



B

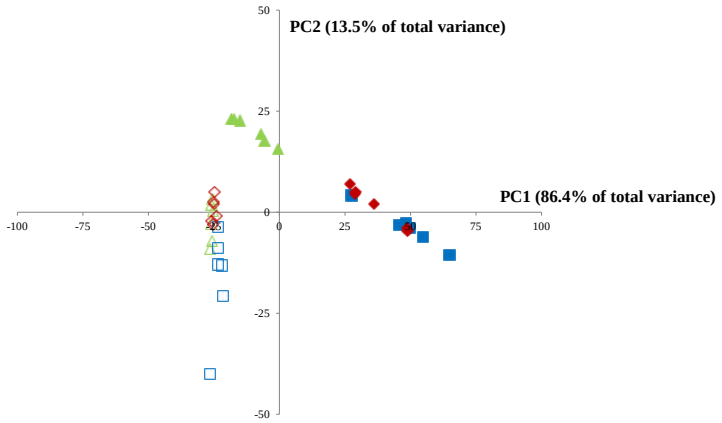


Figure 6.1. *Scores of the principal component analysis (PCA) of metabolite profiles in wild-type roots and leaves under control conditions in Arabidopsis thaliana. The analysis was performed on all metabolites (A) or sugars only (B) that were commonly detected in unexposed roots and leaves over the entire experimental time course (0, 24 and 72 h). Samples were taken at 0 h (roots: open green triangles; leaves: filled green triangles), 24 h (roots: open blue squares; leaves: filled blue squares) and 72 h (roots: open red diamonds; leaves: filled red diamonds). Per time point, 6 biological replicates were analysed.*

For a more thorough analysis, all values were normalised against the mean of the control samples taken at 0 h (set at 1.00) and subjected to a two-way ANOVA analysis per metabolite for roots and leaves separately. In roots, most amino acids decreased while most organic acids increased over time. Moreover,

fructose and glucose levels transiently increased after 24 h in unexposed WT roots (Supplementary Table 6.5A). Due to the higher variance observed between samples in the leaves, less significant differences were obtained. Nonetheless, fructose and glucose displayed an increase after 24 h that was still sustained after 72 h in unexposed leaves. Maltose levels on the other hand significantly dropped after 24 h and were still suppressed after 72 h (Supplementary Table 6.6A).

6.3.2. Cadmium exposure causes major changes in primary metabolite pools in wild-type roots and leaves

In WT roots, 34 out of 35 detected metabolites showed a significant response to Cd after 24 and/or 72 h exposure (Supplementary Table 6.5B). For the roots, PCA revealed that the first and second principal components accounted for 53.7% and 25.2% of the total variance respectively (Figure 6.2A). Of all 34 metabolites found in WT leaf samples, 31 significantly changed in abundance during the experimental time course (Supplementary Table 6.6B). Sample groups derived from plants exposed to 5 or 10 µM Cd after 72 h clearly clustered separately from the remaining sample groups (Figure 6.2B). In this PCA, the first two principal components accounted for respectively 77.1% and 10.5% of the total variance within the leaf dataset (Figure 6.2B). These datasets indicate a fast and large-scale disturbance of primary plant metabolism in response to sublethal Cd exposure in both roots and leaves.

Per metabolite, values were normalised against the mean of the control samples (set at 1.00) per time point (24 and 72 h) and statistically analysed using ANOVA in roots (Supplementary Table 6.5B) and leaves (Supplementary Table 6.6B). For a better overview of the general changes, a schematic metabolic network depicting Cd-induced changes relative to the control is shown for each time point in WT roots (Figure 6.3A) and leaves (Figure 6.3B).

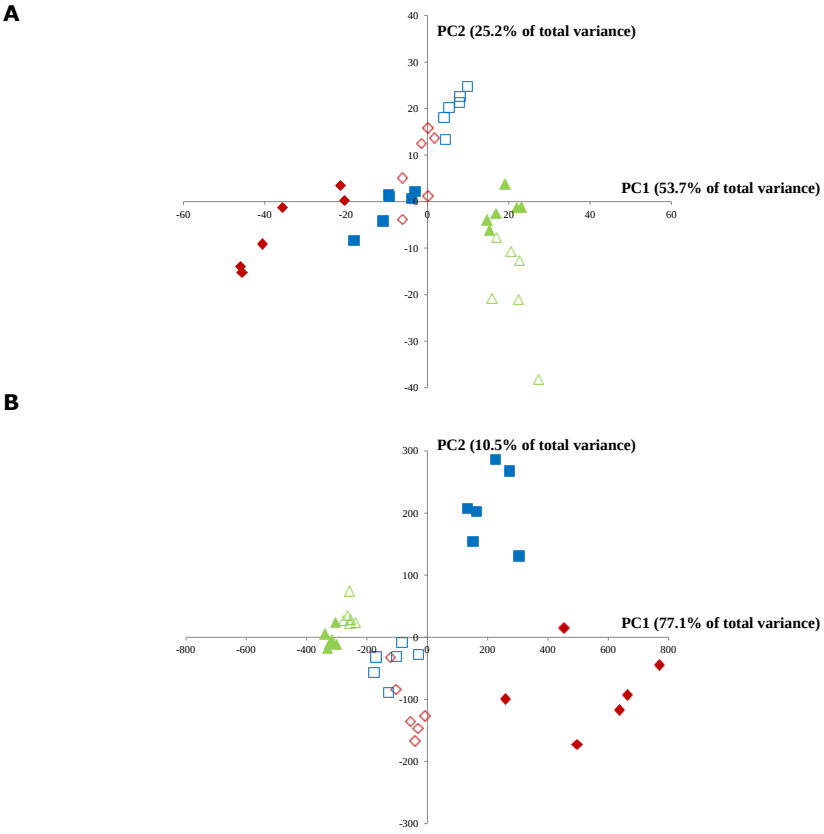


Figure 6.2. *Scores of the principal component analysis (PCA) of metabolite profiles in wild-type roots and leaves under Cd exposure in Arabidopsis thaliana. The analysis was performed on all metabolites detected in roots (A) and leaves (B) of plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Samples for control (green triangles), 5 µM Cd (blue squares) and 10 µM Cd (red diamonds) conditions were taken after 24 h (open symbols) and 72 h (filled symbols). Per condition (tissue, Cd exposure, time point), 6 biological replicates were analysed.*

After 24 h, amino acid levels were generally enhanced in a dose-dependent manner in both tissues (Figure 6.3). Sugars, pyruvate and TCA cycle intermediates on the other hand decreased in roots (Figure 6.3A) while being enhanced (except for pyruvate) in the leaves at this time point (Figure 6.3B). After 72 h, amino acids were still higher under Cd exposure in both roots and leaves (Figure 6.3). As opposed to the previous time point, sucrose and citrate levels were slightly higher after 72 h in Cd-exposed roots as compared to the control. Nonetheless, levels of fructose, glucose, pyruvate and remaining TCA cycle organic acids were still suppressed after 72 h Cd exposure in the roots

(Figure 6.3A) while (except for pyruvate) still accumulated in Cd-exposed leaves in a dose-dependent way (Figure 6.3B). Stress-associated metabolites such as (dehydro)ascorbate, proline, putrescine, raffinose and trehalose increased in abundance in roots after prolonged (72 h) Cd exposure (Figure 6.3A). In the leaves, there was also evidence of changes in the polyamine pathways as putrescine levels highly increased while spermidine decreased in abundance mostly after 72 h exposure to both 5 and 10 µM Cd (Figure 6.3B).

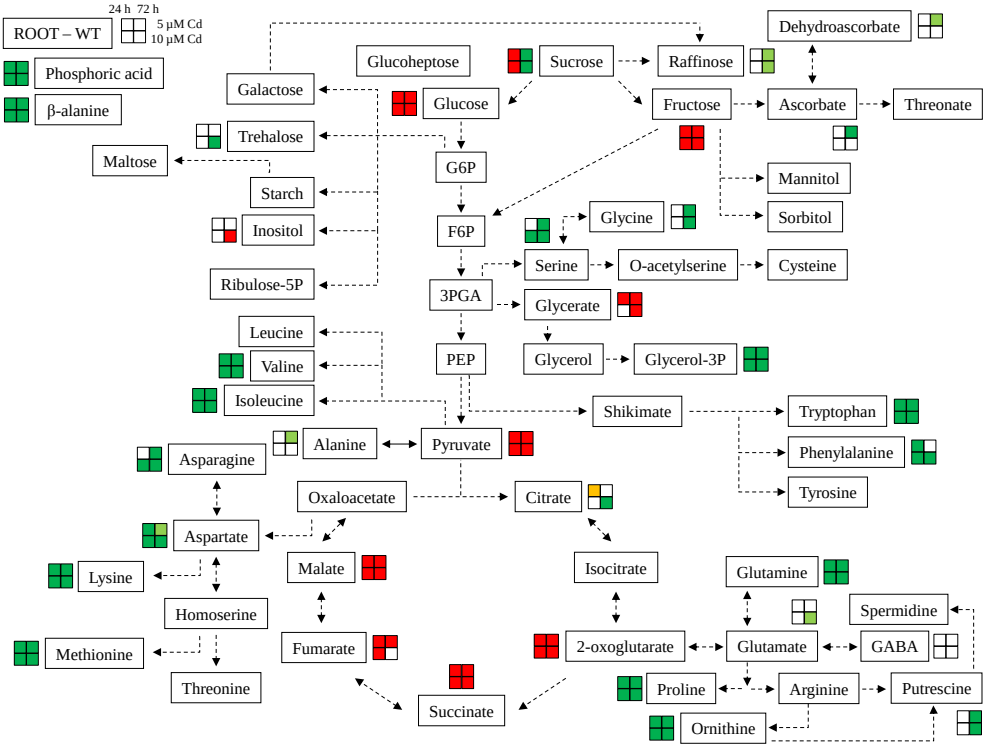


Figure 6.3A. *Schematic overview of primary metabolite abundance changes after 24 and 72 h exposure to 5 or 10 µM $CdSO_4$ in wild-type (WT) Arabidopsis thaliana roots. For each detected metabolite, the colour code in the accompanying square depicts significant Cd-induced increases ($p < 0.05$; $p < 0.01$) and decreases ($p < 0.05$; $p < 0.01$) in abundance relative to the control after 24 (left) and 72 h (right) (two-way ANOVA per time point). Relative changes in abundance and their corresponding significance levels are shown in Supplementary Table 6.5B.*

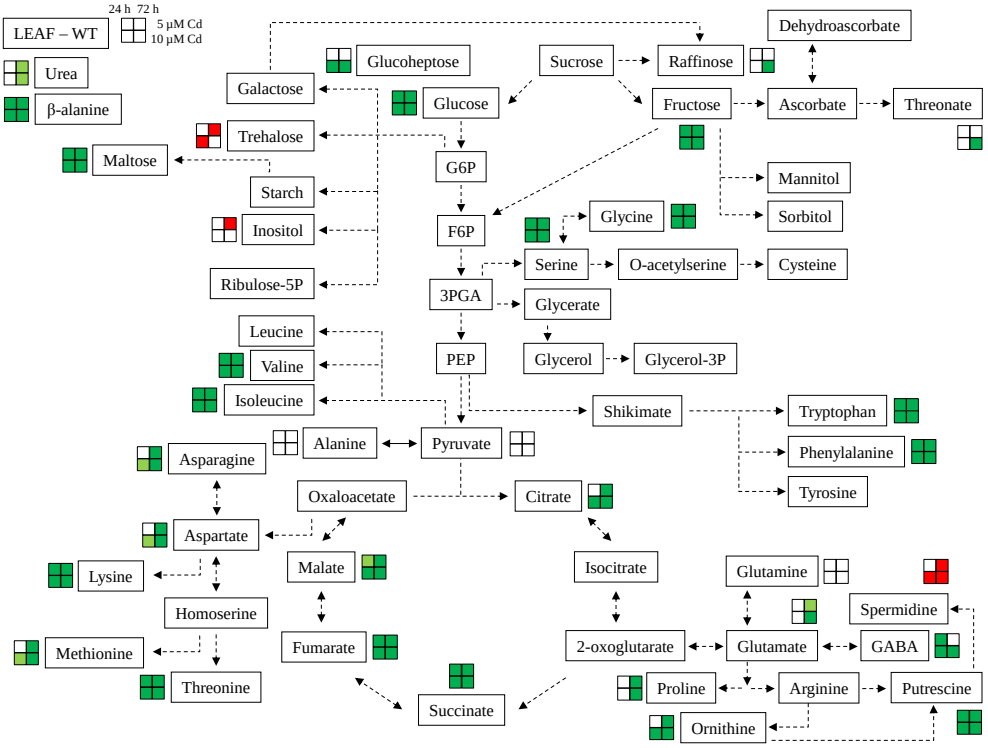


Figure 6.3B. *Schematic overview of primary metabolite abundance changes after 24 and 72 h exposure to 5 or 10 µM $CdSO_4$ in wild-type (WT) Arabidopsis thaliana leaves. For each detected metabolite, the colour code in the accompanying square depicts significant Cd-induced increases ($p < 0.05$; $p < 0.01$) and decreases ($p < 0.01$) in abundance relative to the control after 24 (left) and 72 h (right) (two-way ANOVA per time point). Relative changes in abundance and their corresponding significance levels are shown in Supplementary Table 6.6B.*

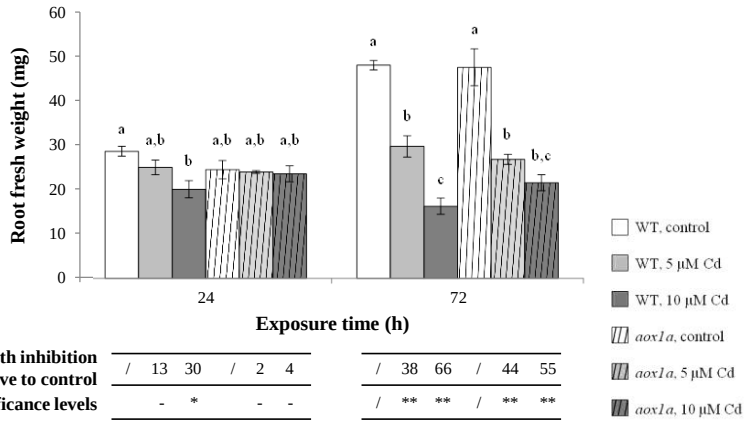
6.3.3. The involvement of mitochondrial AOX1a in Cd stress responses of Arabidopsis thaliana plants

Within the metabolism of plant cells, mitochondria occupy a central position by the presence of the tricarboxylic acid (TCA) cycle, coupling the oxidation of organic acids to ATP synthesis via oxidative phosphorylation. Recently, Vanlerberghe (2013) reviewed the importance of mitochondrial AOX in maintaining metabolic homeostasis during (a)biotic stress in plants. Furthermore, we demonstrated a strong upregulation of the dominant *AOX1a* isoform at the transcript level in both roots and leaves of *A. thaliana* plants exposed to 5 or 10 µM Cd in a kinetic setup (Keunen *et al*. 2013a). Therefore, responses of Cd-exposed WT and *aox1a* knockout plants were compared after 24 and 72 h exposure to both Cd concentrations.

To characterise plant responses at the morphological level, growth was monitored for roots and leaves of both genotypes (Figure 6.4). For the roots, a significantly reduced fresh weight was observed for WT plants exposed to 10 µM Cd after 24 h, which did not occur for *aox1a* knockout roots (Figure 6.4A). Moreover, the dose-dependent reduction in root growth after prolonged (72 h) Cd exposure in WT plants was less pronounced in the mutants (Figure 6.4A). For the leaves, significant differences in fresh weight as compared to unexposed plants were only apparent after 72 h Cd exposure (Figure 6.4B). While Cd caused a dose-dependent inhibition of leaf growth after 72 h in WT plants, this was not significant in *aox1a* knockout mutants (Figure 6.4B).

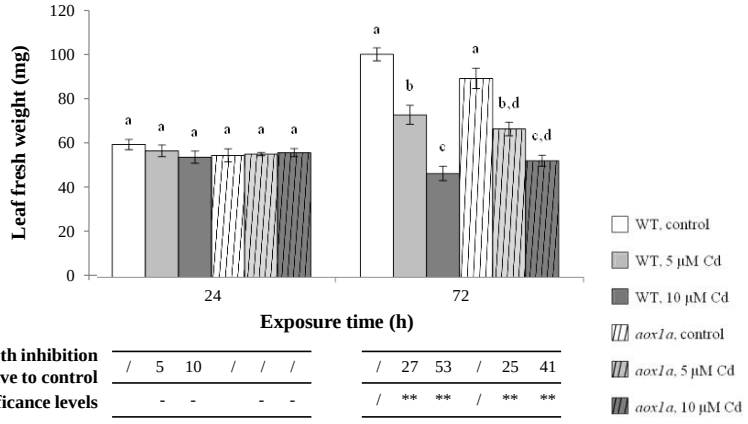
Cadmium is readily taken up by roots and rapidly transported to other plant tissues. The increases in root and leaf Cd content observed after 24 and 72 h in this study were similar to those observed before (Keunen *et al*. 2013a). As no genotype-dependent differences in Cd content were detected (Chapter 5), any differential response between WT and *aox1a* knockout plants is related to a lack of AOX1a function in the latter genotype.

A



% growth inhibition relative to control	/	13	30	/	2	4	/	38	66	/	44	55
Significance levels		-	*		-	-	/	**	**	/	**	**

B



% growth inhibition relative to control	/	5	10	/	/	/	/	27	53	/	25	41
Significance levels		-	-		-	-	/	**	**	/	**	**

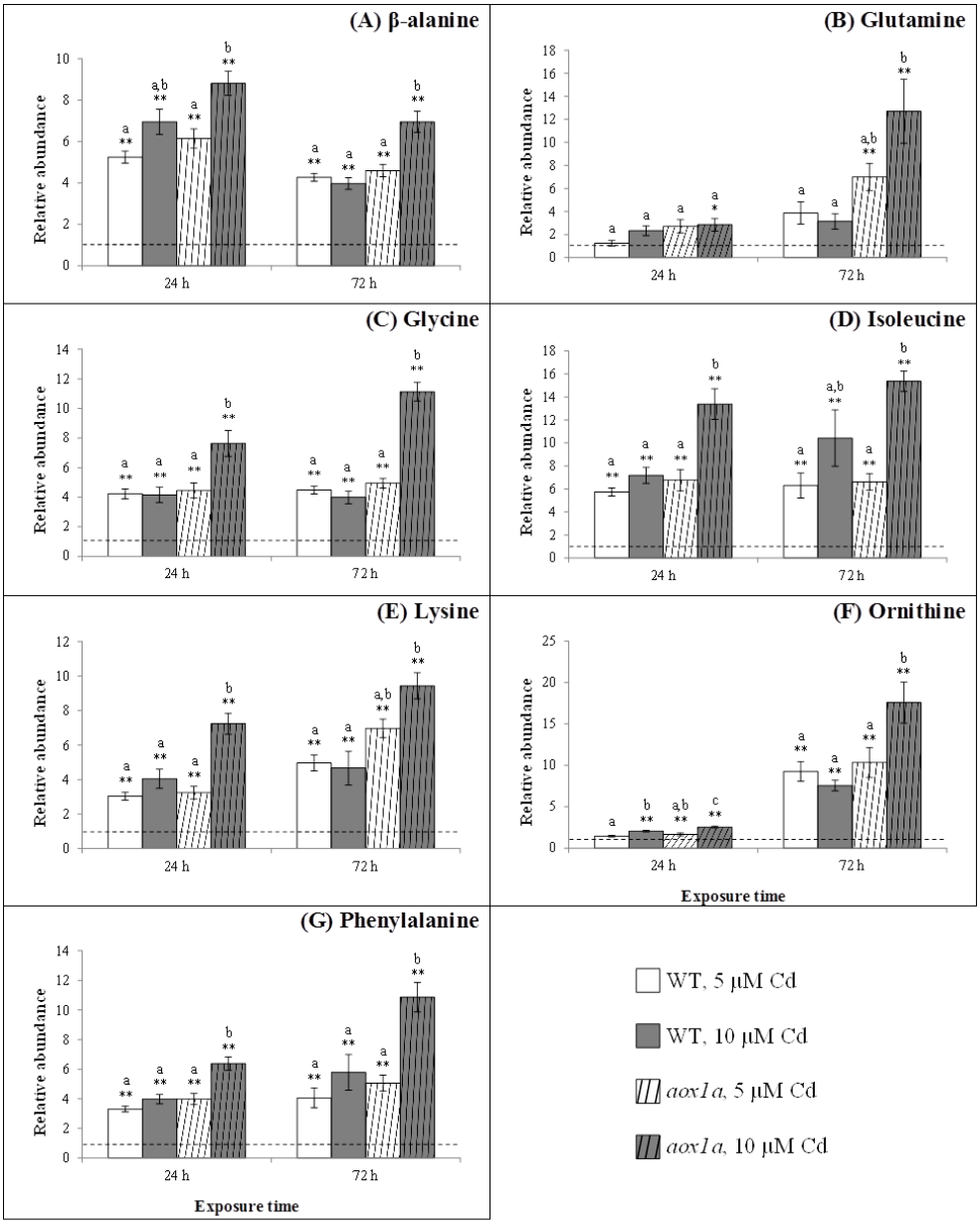
Figure 6.4. *Fresh weight (mg) and growth inhibition (%) of roots (A) and leaves (B) harvested from wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants. The fresh weight was determined for 19-days-old plants exposed to 5 µM (WT: light grey; aox1a: light grey striped) or 10 µM (WT: dark grey; aox1a: dark grey striped) $CdSO_4$ during 24 and 72 h or grown under control conditions (WT: white; aox1a: white striped). For the fresh weight, data are given as the mean ± S.E. of 5 biological replicates, each consisting of at least 10 individual roots or rosettes. Letters a-d represent significant differences within and between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA). For the growth inhibition, data are the mean percentages of inhibition relative to the own unexposed genotype per time point. Significance levels: - no significant difference; * $p < 0.05$ and ** $p < 0.01$ (two-way ANOVA).*

6.3.3.1. Metabolic changes in aox1a knockout in comparison to wild-type plants under control conditions

As for WT plants, metabolites detected in roots of unexposed *aox1a* knockout mutants showed a time-related pattern when comparing samples taken at 0 h and 24 or 72 h later. Initially (at 0 h), GABA levels were lower and pyruvate levels slightly higher in roots of unexposed *aox1a* knockout plants as compared to the WT. However, at later time points, these differences disappeared and no consistent genotype-dependent alterations were observed as for the remaining metabolites under control conditions (Supplementary Table 6.5A). In the leaves, one genotype-dependent difference was related to fructose and glucose levels that increased after 24 h, but only significantly dropped in leaves of *aox1a* knockout plants after 72 h (Supplementary Table 6.6A).

6.3.3.2. Metabolic changes in leaves of Cd-exposed wild-type and aox1a knockout plants

Relative changes in abundance and their significance levels under Cd exposure conditions are shown per genotype and per time point for each metabolite detected in leaves (Supplementary Table 6.6B). In Figure 6.5, only metabolites showing a genotype-specific response to Cd exposure after 24 and/or 72 h are depicted. The levels of several amino acids such as β-alanine, glutamine, glycine, isoleucine, lysine, ornithine, phenylalanine, serine, threonine, tryptophan and valine were consistently increased to a higher extent in leaves of 10 µM Cd-exposed *aox1a* knockout mutants than in WT plants (Figure 6.5A-K). Although this difference occurred after 24 h for several amino acids, it was mostly pronounced after prolonged (72 h) exposure to the highest Cd concentration. Finally, genotype-dependent differences were observed for putrescine and spermidine, intermediates in the stress-related polyamine pathway. After 24 h, putrescine accumulated more in 10 µM Cd-exposed *aox1a* knockout as compared to WT leaves, while the same held true for leaves of mutant plants exposed to both Cd concentrations after 72 h (Figure 6.5L). Whereas spermidine levels were already suppressed after 24 h exposure to 10 µM Cd in leaves of WT plants, leaves of *aox1a* knockout mutants did show a decreased spermidine level but only after prolonged (72 h) Cd exposure (Figure 6.5M).


(A) β-alanine
(B) Glutamine
(C) Glycine
(D) Isoleucine
(E) Lysine
(F) Ornithine
(G) Phenylalanine
Relative abundance
24 h
72 h
Exposure time
WT, 5 µM Cd
WT, 10 µM Cd
aox1a, 5 µM Cd
aox1a, 10 µM Cd

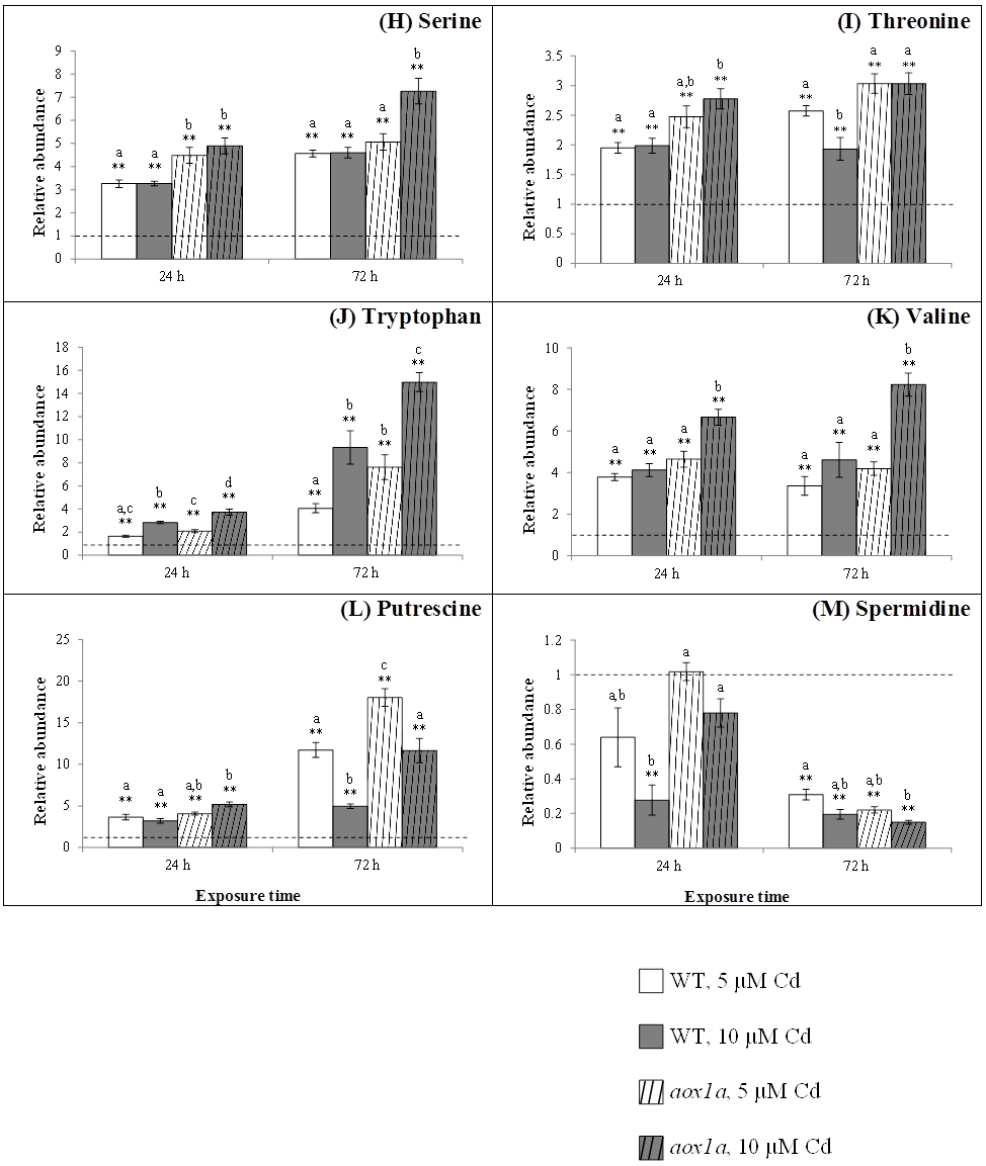


Figure 6.5. *Relative abundance of differentially influenced metabolites in leaves of Cd-exposed wild-type and aox1a knockout Arabidopsis thaliana plants. Metabolite levels were determined via GC-TOF-MS in leaf samples of 19-days-old wild-type (WT) and aox1a knockout (aox1a) plants exposed to 5 µM (WT: white; aox1a: white striped) or 10 µM (WT: grey; aox1a: grey striped) $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 6 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced abundance changes relative to the control are indicated using asterisks: * $p < 0.05$; ** $p < 0.01$. Different letters denote significant differences within and between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA).*

6.3.3.3. Metabolic changes in roots of Cd-exposed wild-type and aox1a knockout plants

In Supplementary Table 6.5B, relative changes in abundance during Cd exposure as compared to the mean of the control samples and their associated significance levels are shown per genotype and per time point for each metabolite detected in roots. In general, Cd-evoked changes did not differ to a great extent between roots of WT and *aox1a* knockout plants. Nonetheless, some genotype-dependent differences were observed, both after 24 and 72 h (Figure 6.6). Sugars (fructose, glucose and sucrose) dropped to a lower level in *aox1a* knockout as compared to WT roots after 24 h exposure to the highest Cd concentration, albeit only significant for sucrose (Figure 6.6A). After 72 h exposure to 10 µM Cd, *aox1a* knockout mutant roots accumulated significantly less citrate (Figure 6.6B) and fumarate (Figure 6.6C) as compared to the WT. On the other hand, glutamine levels (Figure 6.6D) were higher in 10 µM Cd-exposed roots of *aox1a* knockout than in WT plants after 72 h. Finally, levels of ascorbate (AsA, Figure 6.6E) and its oxidised form dehydroascorbate (DHA, Figure 6.6F) significantly increased in *aox1a* mutant roots only after 24 h exposure to 10 µM Cd.

6.3.4. The relationship between AOX1a and antioxidative defence mechanisms in roots of Cd-exposed Arabidopsis thaliana plants

A lack of functional AOX1a seems to impact the response of the important antioxidant metabolite AsA and its oxidised form DHA in Cd-exposed roots (cfr. *supra*). Therefore, we determined if and how AOX1a affects the Cd-induced oxidative challenge at the transcript and enzymatic level in *A. thaliana* roots.

Relative expression levels of genes encoding marker for ROS-induced gene expression (Gadjev *et al*. 2006), different ROS producing, both mitochondrial and other ROS scavenging enzymes were determined in roots of WT and *aox1a* knockout plants exposed to 5 or 10 µM Cd during 24 and 72 h (Supplementary Table 6.7), without differences in transcript levels between unexposed genotypes. Transcript levels of all hallmark genes were increased similarly in both genotypes upon Cd exposure, except for the TIR-class gene (*AT1G57630*). This gene was induced to a greater extent in 10 µM Cd-exposed *aox1a* knockout than in WT roots after 24 h (Supplementary Table 6.7).

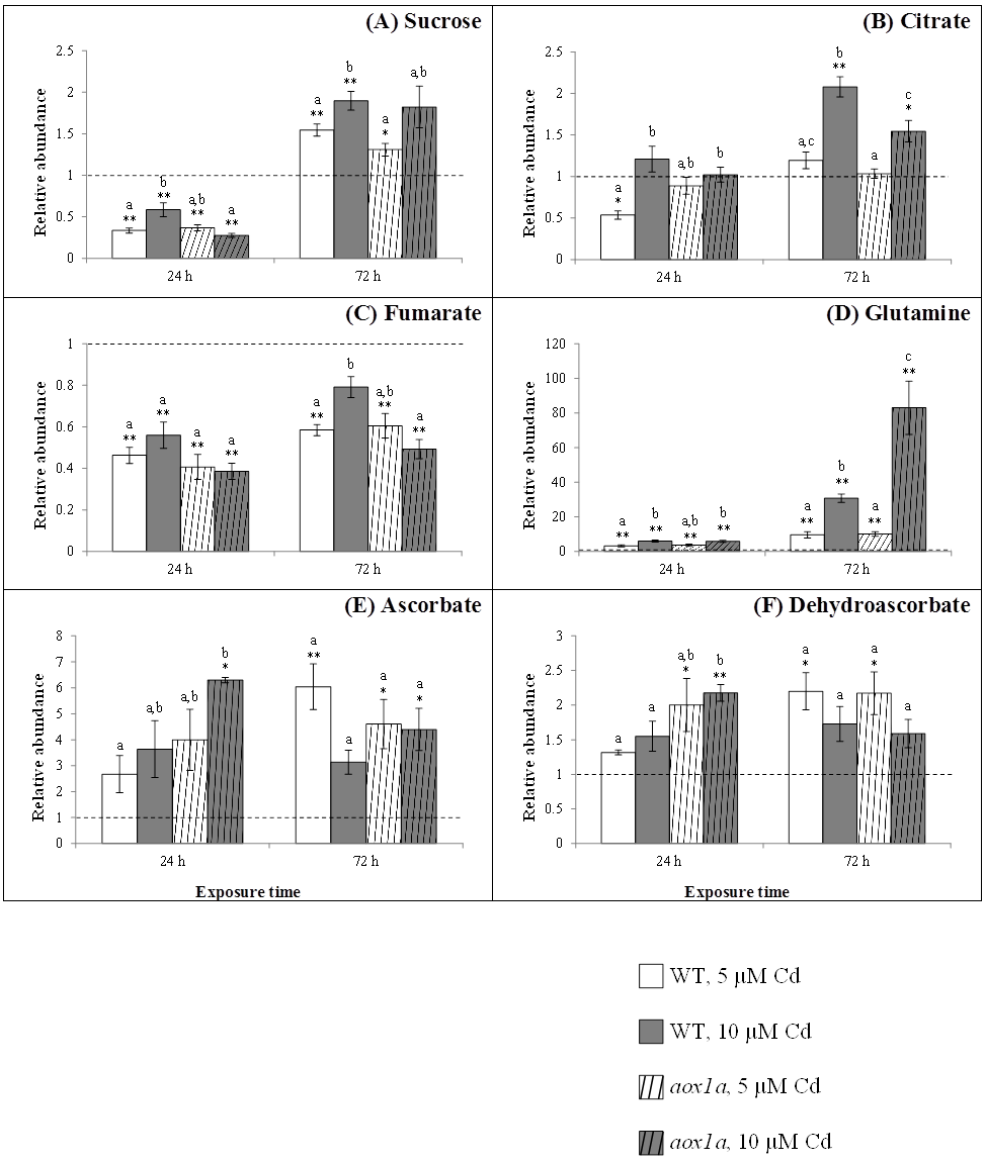


Figure 6.6. *Relative abundance of differentially influenced metabolites in Cd-exposed roots of wild-type and aox1a knockout Arabidopsis thaliana plants. Metabolite levels were determined via GC-TOF-MS in root samples of 19-days-old wild-type (WT) and aox1a knockout (aox1a) plants exposed to 5 µM (WT: white; aox1a: white striped) or 10 µM (WT: grey; aox1a: grey striped) $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 6 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced abundance changes relative to the control are indicated using asterisks: * $p < 0.05$; ** $p < 0.01$. Different letters denote significant differences within and between both genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA).*

In general, for WT plants, responses of pro- and antioxidative genes coincided with our earlier work as described by Cuypers *et al*. (2011), Keunen *et al*. (2013a, 2013b) and Jozefczak *et al*. (2014). Expression levels of ROS producing lipoxygenase (*LOX*) isoforms 1, 4 and 6 and respiratory burst oxidase homologue (*RBOH*) isoforms D and F were increased, while those of *LOX5* and *RBOHC* were suppressed after Cd exposure (Supplementary Table 6.7). Except for a higher induction of *LOX6* after 24 h and of *RBOHD* after 72 h in 10 µM Cd-exposed *aox1a* knockout as compared to WT roots, no genotype-dependent effects could be observed (Supplementary Table 6.7). Expression of antioxidative genes such as *CAT1* and *CAT3*, iron SOD1 (*FSD1*) and *GR1* were increased after Cd exposure in both genotypes. Ascorbate peroxidase 1 (*APX1*), *CAT2*, copper/zinc SOD1 and 2 (*CSD1/2*) gene expression diminished similarly in Cd-exposed WT and *aox1a* knockout roots (Supplementary Table 6.7). Regarding mitochondrial antioxidative genes, responses in WT roots were generally similar as described before (Keunen *et al*. 2013a), with a slightly higher expression of peroxiredoxin in Cd-exposed *aox1a* knockout than in WT roots after 72 h exposure (Supplementary Table 6.7).

As transcriptional pro- and antioxidative responses in roots were generally similar for WT and *aox1a* knockout mutants, capacities of antioxidative and NAD(P)H-dependent enzymes were measured in roots of both genotypes exposed to 5 or 10 µM Cd during 24 and 72 h (Table 6.1). Except for CAT, SPOD and GDH, capacities were significantly enhanced after 72 h exposure to 10 µM Cd in roots of *aox1a* knockout plants alone (Table 6.1). The capacity of NADP-ME was increased to a greater extent in 10 µM Cd-exposed *aox1a* mutant as compared to WT roots after both 24 and 72 h (Table 6.1).

Table 6.1. *Root capacities of antioxidative and NAD(P)H-dependent dehydrogenases in Arabidopsis thaliana. Capacities were determined in roots of 19-days-old wild-type (WT) and aox1a knockout (aox1a) plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 4 biological replicates expressed in U g^{-1} fresh weight for SOD and in mU g^{-1} fresh weight for all other enzymes. Significant Cd-induced capacity increases within each genotype are indicated with colour shading: $p < 0.05$; $p < 0.01$, while differences between both genotypes are indicated with asterisks ($p < 0.05$) (two-way ANOVA per time point). Abbreviations: SOD, superoxide dismutase; CAT, catalase, GR, glutathione reductase; GPOD, guaiacol peroxidase; SPOD, syringaldazine peroxidase; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glutamate dehydrogenase; ICDH, isocitrate dehydrogenase; ME, NADP-malic enzyme.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Antioxidative enzymes					
	Control	95.00 ± 19.38	170.48 ± 16.91	52.50 ± 13.90	103.70 ± 21.02
SOD	**5 µM Cd**	122.12 ± 21.19	96.66 ± 28.61	135.26 ± 20.32	143.44 ± 23.84
	10 µM Cd	153.83 ± 39.51	132.42 ± 34.12	148.98 ± 17.06	276.02 ± 29.97*
	Control	20.67 ± 5.73	16.13 ± 3.27	18.01 ± 5.63	28.89 ± 6.39
CAT	**5 µM Cd**	14.37 ± 1.34	18.50 ± 2.67	25.93 ± 2.59	34.89 ± 6.80
	10 µM Cd	19.00 ± 4.59	38.50 ± 15.40	31.38 ± 7.47	54.57 ± 12.30
	Control	169.57 ± 38.53	191.25 ± 11.80	113.78 ± 24.06	160.51 ± 31.09
GR	**5 µM Cd**	195.62 ± 22.84	150.67 ± 28.59	151.08 ± 30.07	219.45 ± 55.25
	10 µM Cd	171.15 ± 5.66	231.45 ± 9.99	254.64 ± 13.30	547.57 ± 81.17*
	Control	1376.64 ± 313.79	1733.87 ± 129.79	897.23 ± 303.24	1694.18 ± 375.61
GPOD	**5 µM Cd**	1600.21 ± 246.55	989.23 ± 277.99	1540.77 ± 388.84	2190.52 ± 565.88
	10 µM Cd	1420.94 ± 11.17	1748.13 ± 15.94	2182.64 ± 176.56	3899.92 ± 318.67
	Control	4420.16 ± 1254.88	4661.64 ± 407.01	2594.31 ± 706.74	4291.36 ± 1335.51
SPOD	**5 µM Cd**	4370.31 ± 856.57	2189.20 ± 474.58	2788.09 ± 809.95	3718.63 ± 1055.24
	10 µM Cd	2201.40 ± 304.55	4082.12 ± 255.61	3816.37 ± 598.74	8652.21 ± 2553.20

Table 6.1. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
NAD(P)H-dependent dehydrogenases					
	Control	15.04 ± 2.35	28.31 ± 5.72	18.41 ± 4.24	14.61 ± 3.72
G6PDH	**5 µM Cd**	20.93 ± 5.44	16.05 ± 1.60	17.46 ± 4.46	23.00 ± 9.41
	10 µM Cd	16.07 ± 4.79	26.25 ± 4.73	27.41 ± 7.67	70.80 ± 15.07
	Control	24.11 ± 2.37	33.95 ± 1.31	25.16 ± 5.48	37.03 ± 5.22
GDH	**5 µM Cd**	29.82 ± 4.92	37.18 ± 5.40	38.93 ± 5.72	50.21 ± 12.78
	10 µM Cd	21.97 ± 0.57	33.34 ± 8.85	50.00 ± 9.40	56.95 ± 2.89
	Control	223.77 ± 59.98	218.62 ± 23.72	63.87 ± 4.84	169.91 ± 40.02
ICDH	**5 µM Cd**	277.28 ± 55.85	176.62 ± 57.73	214.14 ± 60.35	288.71 ± 81.98
	10 µM Cd	204.24 ± 3.98	307.38 ± 8.39	302.70 ± 26.88	669.07 ± 128.00
	Control	172.74 ± 38.93	189.28 ± 19.65	85.83 ± 25.86	145.74 ± 33.87
ME	**5 µM Cd**	295.72 ± 46.84	178.18 ± 57.75	297.37 ± 77.22	366.02 ± 110.31
	10 µM Cd	259.35 ± 21.87	450.03 ± 33.15*	459.50 ± 28.22	1058.77 ± 202.97*

Finally, *AOX1a* gene expression levels were determined in roots of WT and GSH-deficient *cad2-1*, *pad2-1* and *rax1-1* plants exposed to 5 µM Cd during 24 and 72 h (Figure 6.7). Reduced GSH levels caused a strong upregulation of *AOX1a* expression during Cd exposure at both time points. Roots of *pad2-1* mutants displayed the strongest *AOX1a* induction as compared to the other genotypes, although its *AOX1a* expression level was not significantly different from that in *cad2-1* after 24 h (Figure 6.7).

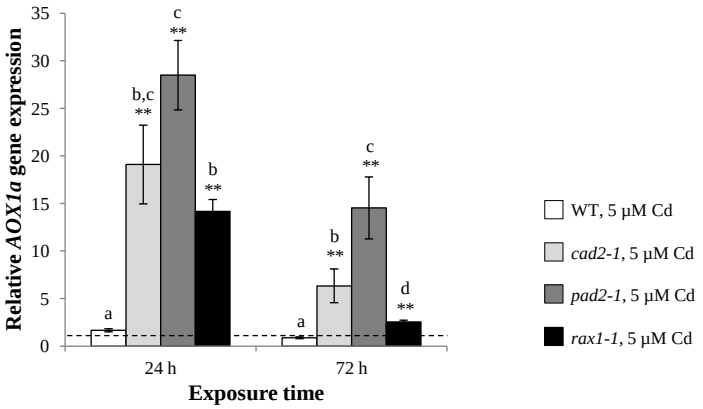


Figure 6.7. *Relative root AOX1a transcript levels in wild-type and GSH-deficient Arabidopsis thaliana plants. Transcript levels were measured via quantitative real-time PCR in root samples of 19-days-old plants exposed to 5 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Genotypes: wild-type (white), cad2-1 (light grey), pad2-1 (dark grey) and rax1-1 plants (black). Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed genotype set at 1.00 (dashed line). Within each genotype and time point, significant Cd-induced expression changes relative to the control are indicated using asterisks: * $p < 0.05$; ** $p < 0.01$. Different letters denote significant differences between genotypes ($p < 0.05$), tested within each exposure time (two-way ANOVA).*

6.4. DISCUSSION

To obtain optimal and controllable growth conditions for *A. thaliana*, a hydroponic system has been critically evaluated and optimised in our group (Smeets *et al*. 2008a). This system enables separate harvesting of root and leaf samples to study plant responses using sensitive parameters at the biochemical and molecular level. When comparing metabolic profiles of roots and leaves sampled from WT control plants over a time period ranging from 0 to 72 h, subtle to substantial differences could be observed mostly for sugars in both tissues (Figure 6.1, Supplementary Tables 6.5A and 6.6A).

As carbohydrates generated by photosynthesis are the building units and energy providers to produce and maintain plant biomass, these changes are mirrored by a vast increase in root and leaf fresh weight of unexposed plants during the experimental time course (Figure 6.4). Moreover, as metabolite levels alter over time (e.g. amino acids in roots), it is important to normalise changes induced by Cd exposure to the mean of the control samples per time point. In this way, an objective interpretation of how Cd affects basal metabolism as compared to unexposed plants is allowed.

6.4.1. Differential metabolic responses in roots and leaves of wild-type Cd-exposed Arabidopsis thaliana plants

Sarry *et al*. (2006) have reported the immediate (24 h) metabolic response of *A. thaliana* cells exposed to $CdCl_2$ concentrations ranging from 0.5 to 200 µM. However, it was shown that the response of plant tissues such as roots can differ substantially from that of heterotrophic cell cultures under similar stress conditions (Lehmann *et al*. 2009). As opposed to a previous study by Sun *et al*. (2010) characterising metabolic changes in whole plants exposed during two weeks, we studied the metabolic impact of acute (24 h) and prolonged (72 h) sublethal Cd exposure in roots and leaves separately. An overview of how Cd affects the primary metabolism in both tissues is presented in Figure 6.3.

In our hydroponic cultivation system, *A. thaliana* plants are exposed to 5 or 10 µM $CdSO_4$ via the roots. Levels of sugars (sucrose, fructose and glucose), pyruvate and TCA cycle intermediates dropped significantly in roots, while being increased in leaves of Cd-exposed plants after 24 h exposure. Concurrently, most amino acids showed an enhanced level in both Cd-exposed roots and leaves as compared to the control (Figure 6.3, Supplementary Tables 6.5B and 6.6B). As roots represent the first contact site for Cd within the plant, defence mechanisms are instantly activated (Jozefczak *et al*. 2014). Expression levels of genes encoding proteins involved in transcription, translation, antioxidative defence and repair mechanisms were demonstrated to rapidly increase during Cd exposure using different experimental setups (Herbette *et al*. 2006, Smeets *et al*. 2008b). Moreover, the protein abundance of these genes altered in a Cd-exposed *A. thaliana* cell culture within 24 h (Sarry *et al*. 2006). Both transcription and translation processes require energy, which is also reflected by

the metabolic profile obtained for Cd-exposed roots (Figure 6.3A). These results point towards an increased glycolytic flux and TCA cycle activity, potentially to support enhanced amino acid and protein synthesis. On the other hand, sugars and TCA cycle intermediates were increased after 24 h Cd exposure in leaves. The lack of an initial decrease in the level of these metabolites as compared to the response in roots might be explained by a direct and strong accumulation of maltose in Cd-exposed leaves, mainly after 24 h (Figure 6.3B, Supplementary Table 6.6B). Maltose is the principal product of starch degradation in *A. thaliana* leaves (Niittylä *et al*. 2004, Weise *et al*. 2004). During Cd exposure, it thus appears that leaves mainly utilise their starch reserves to provide energy for stress defence. Using electron microscopy, we have demonstrated that leaves of *A. thaliana* plants chronically exposed to 5 µM Cd indeed contain less starch granules as compared to leaves of control plants (Van Belleghem 2007). A second possible mechanism underlying the differential metabolic response in both tissues might be Cd-induced root-to-shoot signalling. As roots are the first contact point for Cd, signalling pathways might be activated and prime other plant parts to prepare for future stress conditions (Smeets *et al*. 2008b). The nature of this signal is currently unknown, but ROS are tempting candidates as suggested before (Capone *et al*. 2004, Cuypers *et al*. 2011). After 72 h, sucrose and citrate levels recovered from the initial drop after 24 h in Cd-exposed roots (Figure 6.3A). This suggests that under prolonged exposure, energy is (again) provided by the leaves to support stress defence throughout the entire plant.

Several known stress-related metabolites displayed an altered abundance in both roots and leaves of Cd-exposed *A. thaliana*. For example, levels of raffinose and trehalose increased in 10 µM Cd-exposed roots after 72 h (Figure 6.3A), while trehalose decreased and raffinose increased in Cd-exposed leaves (Figure 6.3B). Both sugars protect against hydroxyl radicals (Nishizawa *et al*. 2008, Lunn *et al*. 2014), the production of which is also associated with Cd-induced oxidative stress (Cuypers *et al*. 2012). As reviewed by Obata and Fernie (2012), raffinose accumulates especially at later stages of stress treatment. This is also the case in our study, with significantly increased raffinose levels only appearing after 72 h Cd exposure in both roots and leaves (Figure 6.3). Recently, Lunn *et al*. (2014) reviewed the role of trehalose in plants and proposed a bidirectional relationship with sucrose. The observed

decrease in leaf trehalose levels during Cd exposure points towards an increase in sucrose to support respiration, as evidenced by the increased abundance of TCA cycle intermediates (Figure 6.3B). Moreover, sucrose can also be exported to sink organs, coinciding with increasing levels after 72 h exposure to both 5 and 10 µM Cd after the initial drop in roots (Figure 6.3A).

Finally, our study has evidenced that sublethal Cd exposure affects the polyamine pathways in roots and leaves as supported by increased putrescine levels (Figure 6.3). Accumulation of polyamines is associated with increased tolerance to environmental stresses such as drought, cold, salinity and metal exposure (Gill and Tuteja 2010). It is highly probable that polyamines effectively stabilise and protect membranes from Cd-induced oxidative damage (Geuns *et al*. 1997, Sharma and Dietz 2006). Moreover, the decreased spermidine levels observed in Cd-exposed leaves (Figure 6.3B) correspond to earlier observations in mung bean seedlings (Geuns *et al*. 1997) and sunflower leaf discs (Groppa *et al*. 2003) exposed to Cd. Spermidine might decrease because of a Cd-mediated increase in ethylene production, diverting S-adenosylmethionine precursors away from polyamine biosynthesis (Groppa *et al*. 2003). Recently, Schellingen *et al*. (2014) demonstrated that Cd exposure leads to ethylene production under our experimental conditions, thereby supporting the hypothesis of Groppa *et al*. (2003). Finally, a declined spermidine titer is correlated with leaf senescence (Galston and Sawhney 1990) as is also postulated for Cd exposure (Geuns *et al*. 1997, Sandalio *et al*. 2001).

6.4.2. Mitochondria and AOX1a are involved in Cd stress responses of Arabidopsis thaliana plants

Comparing the metabolic profiles of Cd-exposed roots and leaves, a clear difference was manifested at the level of the TCA cycle (Figure 6.3). While organic acids mainly decreased in roots (Figure 6.3A), the opposite was true in leaves of *A. thaliana* plants exposed to 5 or 10 µM Cd during 24 and 72 h (Figure 6.3B). These results suggest a differential response to Cd at the mitochondrial level, pointing towards an altered supply or demand for carbon skeletons, reducing power and ATP during Cd exposure in both tissues. Moreover, plants are equipped with the alternative pathway of electron transport mediated by AOX. As this pathway adjusts the degree of coupling between carbon

metabolism and electron transport, the flexibility of the respiratory system is increased (Vanlerberghe *et al*. 2009). The latter might be particularly helpful during stress conditions such as Cd exposure, when plant metabolism must be reconfigured to maintain metabolic homeostasis and the production of stress-ameliorating components.

Previously, we have demonstrated the involvement of the dominant AOX1a isoform in *A. thaliana* root and leaf responses to Cd at transcript and protein levels (Keunen *et al*. 2013a). Moreover, AOX1a was shown to modulate the oxidative challenge during moderate Cd exposure in *A. thaliana* leaves (Chapter 5). Therefore, Cd responses of WT plants were compared to those of plants lacking functional AOX1a. Cadmium uptake and changes in fresh weight were generally similar for both genotypes (Figure 6.4). Growth of both genotypes was generally similar under control conditions, supporting the view that a lack of AOX1a mainly affects plant stress responses without major consequences for normal plant physiology (Vanlerberghe *et al*. 2009). Similarly, metabolic profiles of both genotypes under control conditions were highly comparable in both roots and leaves (Supplementary Tables 6.5A and 6.6A). However, whereas fructose and glucose levels increased after 24 h in both genotypes, it only dropped significantly in leaves of *aox1a* knockout plants (Supplementary Table 6.6A). Although this might reflect the need for built-in flexibility by AOX in the respiratory metabolism (Vanlerberghe *et al*. 2009), plant growth was not compromised (Figure 6.4).

6.4.3. Differential metabolic responses in leaves of wild-type and aox1a knockout plants exposed to Cd

Regarding the metabolic profiles obtained for leaves of WT and *aox1a* knockout plants exposed to Cd, differences were observed for the stress-related polyamine pathway. Changes in this pathway are intimately related to the production of ethylene (Groppa *et al*. 2003). As discussed before, Cd exposure was shown to evoke ethylene biosynthesis under our experimental conditions (Schellingen *et al*. 2014). This explains the observed decrease in spermidine levels (Figure 6.5M), while its precursor putrescine increased during Cd exposure in the leaves (Figure 6.5L). Ethylene was shown to induce *AOX1a* transcription in Cd-exposed *A. thaliana* leaves, while AOX1a was suggested to exert negative

feedback on ethylene biosynthesis and/or signalling (Chapter 5). The observed differences in putrescine and spermidine levels in *aox1a* knockout as compared to WT leaves might also be related to an altered ethylene production in the absence of functional AOX1a. This further supports a link between ethylene and AOX and the involvement of feedback mechanisms during sublethal Cd exposure in the leaves as suggested before (Chapter 5).

In both WT and *aox1a* knockout plants, Cd exposure caused a significant accumulation of almost all detected amino acids in the leaves, either after 24 or 72 h (Figure 6.3B, Supplementary Table 6.6B). As discussed by Obata and Fernie (2012), this accumulation might be related to increased amino acid synthesis as well as enhanced protein degradation. Assuming the first scenario, decreases in sugars and TCA cycle intermediates are to be expected to provide energy. As exactly the opposite occurred in Cd-exposed leaves (Figure 6.3B), evidence is pointing towards increased protein degradation under Cd exposure.

As reviewed by DalCorso *et al*. (2008), photosynthesis is compromised by Cd exposure. Therefore, the increased level of amino acids in Cd-exposed leaves (Figure 6.3B) might reflect the need for alternative respiratory substrates as proposed by Araújo *et al*. (2011). Next to lipids and chlorophyll, proteins might be degraded into amino acids that directly or indirectly (via the TCA cycle) fuel electrons to the mitochondrial ETC under conditions altering carbohydrate supply. This is supported by elevated levels of proteins involved in proteolysis in leaves of Cd-exposed *A. thaliana* plants (Semane *et al*. 2010, Dupae *et al*. 2014).

For 11 out of 18 detected amino acids increasing in leaves of Cd-exposed plants, a genotype-dependent response could be observed (Figure 6.5). After 24 and mainly 72 h exposure to 10 µM Cd, the abundance of these amino acids was increased to a higher extent in leaves of *aox1a* knockout as compared to WT plants (Figure 6.5A-K). Especially lysine (Figure 6.5E), branched chain (isoleucine and valine, Figure 6.5D and 6.5K) and aromatic amino acids (phenylalanine and tryptophan, Figure 6.5G and 6.5J) accumulated more in the absence of functional AOX1a, and typically these amino acids are used in the degradation-respiratory pathway (Araújo *et al*. 2010, 2011, Obata and Fernie 2012). This suggests that AOX1a might be required to allow increased respiratory flux dependent on amino acid degradation in Cd-exposed leaves.

As suggested by Araújo *et al*. (2010), the role of this alternative respiration should be further explored under environmental stresses such as Cd exposure.

6.4.4. Differential metabolic responses in roots of Cd-exposed wild-type and aox1a knockout plants

After 24 h, root metabolism was strongly perturbed by Cd exposure as evidenced by decreases in sucrose (Figure 6.6A), citrate (Figure 6.6B) and fumarate (Figure 6.6C). As they returned to control levels or even increased after 72 h Cd exposure (Figure 6.6), we suggest that root metabolism was acutely changed but at least partly reconfigured after prolonged (72 h) exposure to Cd in WT plants. However, this pattern changed in roots of *aox1a* knockout plants, with either a stronger decrease after 24 h as for sucrose (Figure 6.6A) or a smaller increase after 72 h exposure to 10 µM Cd as for citrate (Figure 6.6B) and fumarate (Figure 6.6C). These results implicate the involvement of AOX1a in Cd stress responses at the metabolic level in roots in our experimental time frame.

Other genotype-specific alterations in the metabolic profile of Cd-exposed WT versus *aox1a* knockout roots were all related to metabolites involved in antioxidative defence. For example, glutamine levels were increased to a higher extent in roots of *aox1a* knockout plants exposed to 10 µM Cd after 72 h as compared to WT roots (Figure 6.6D). Glutamine is converted to glutamate, which is one of the building blocks for GSH biosynthesis. The conversion step between glutamine and glutamate is executed by glutamine synthetase, and its protein abundance was demonstrated to increase during Cd exposure (Sarry *et al*. 2006, Semane *et al*. 2010). Higher levels of glutamine in roots of *aox1a* knockout as compared to WT plants might reflect a decreased conversion to glutamate and thus GSH in the absence of functional AOX1a. Jozefczak *et al*. (2014) have attributed an important role to GSH as antioxidative as well as Cd-chelating compound in Cd-exposed *A. thaliana* roots. Therefore, *aox1a* knockout plants might experience higher stress levels under Cd exposure.

6.4.5. A reciprocal relationship between AOX1a and antioxidative defence mechanisms in roots of Cd-exposed Arabidopsis thaliana plants

Results of the metabolic profiling suggest that AOX1a is closely related to the acute antioxidative response elicited by Cd in *A. thaliana* roots. Therefore, we determined if and how AOX1a affects the Cd-induced oxidative challenge at additional cellular functional levels in *A. thaliana* roots. Not at transcriptional, but at enzymatic capacity level, differences were noted when determining the effects of Cd on the capacity of antioxidative and NAD(P)H-dependent enzymes (Supplementary Table 6.7, Table 6.1). For 6 out of 9 enzymes measured, capacities increased to a higher extent in *aox1a* knockout as compared to WT roots after 72 h exposure to 10 µM Cd (Table 6.1). These results further support our hypothesis that plants without functional AOX1a suffer from increased (oxidative) stress during Cd exposure. Moreover, as GR capacity was enhanced to a higher extent in roots of *aox1a* knockout as compared to WT plants exposed to 10 µM Cd for 72 h (Table 6.1), the potential relationship between AOX1a and GSH is further underlined.

To explore the potential association between AOX1a and GSH using an opposite approach, the induction of *AOX1a* expression was monitored in roots of Cd-exposed WT plants and GSH-deficient *cad2-1*, *pad2-1* and *rax1-1* mutants (Figure 6.7). The expression of *AOX1a* was induced to a far greater extent in all Cd-exposed GSH-deficient as compared to WT plants. Because of its thiol group and as precursor for PCs, GSH is crucial for chelation and vacuolar sequestration of Cd (Rauser 2001). Using a kinetic exposure setup (2, 24, 48 and 72 h exposure to 5 or 10 µM Cd), Jozefczak *et al*. (2014) recently demonstrated that roots of *A. thaliana* immediately synthesise PCs consuming GSH. In this way, the antioxidant role of GSH is compromised leading to an increased oxidative challenge in Cd-exposed roots. Following this rationale, it is assumed that GSH-deficient mutants suffer from increased (oxidative) stress and therefore induce AOX1a transcription to a higher extent, at least in roots (Figure 6.7). In the future, more research should be devoted to the emerging link between mitochondria, AOX and GSH during sublethal Cd exposure in *A. thaliana*.

6.4.6. Concluding remarks

In conclusion, our study reports on Cd-induced changes in root and leaf primary metabolism, with sugars and organic acids responding oppositely in both tissues of WT *A. thaliana* plants. Whereas a lack of AOX1a does not impact primary metabolism under control conditions, it causes differential responses to sublethal Cd exposure. In leaves, our results confirm the previously suggested link between AOX and Cd-induced ethylene production. Furthermore, AOX1a might allow increased respiratory flux dependent on amino acid degradation. Finally, a reciprocal relationship is suggested between AOX1a and antioxidative defence mechanisms, in particular GSH, at different biological levels in Cd-exposed *A. thaliana* roots.

REFERENCES

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C. and Ecker J.R. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*, **64**, 5245–5250.

Araújo, W.L., Ishizaki, K., Nunes-Nesi, A., Larson, T.R., Tohge, T., Krahnert, I., Witt, S., Obata, T., Schauer, N., Graham, I.A., Leaver C.J. and Fernie, A.R. (2010) Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of *Arabidopsis* mitochondria. *Plant Cell*, **22**, 1549–1563.

Araújo, W.L., Tohge, T., Ishizaki, K., Leaver, C.J. and Fernie, A.R. (2011) Protein degradation – an alternative respiratory substrate for stressed plants. *Trends Plant Sci*, **16**, 489–498.

Ball, L., Accotto, G.P., Bechtold, U., Creissen, G., Funck, D., Jimenez, A., Kular, B., Leyland, N., Mejia-Carranza, J., Reynolds, H., Karpinski, S. and Mullineaux, P.M. (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in Arabidopsis. *Plant Cell*, **16**, 2448–2462.

Bergmeyer, H.U., Gawenn, K. and Grassl, M. (1974) Enzymes as biochemical reagents, in: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 425–522.

Bi, Y.H., Chen, W.L., Zhang, W.N., Zhou, Q., Yun L.J. and Xing, D. (2009) Production of reactive oxygen species, impairment of photosynthetic function and dynamic changes in mitochondria are early events in cadmium-induced cell death in *Arabidopsis thaliana*. *Biol Cell*, **101**, 629–643.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) The MIQE guidelines: Minimum Information for publication of Quantitative real-time PCR Experiments. *Clin Chem*, **55**, 611–622.

Capone, R., Tiwari, B.S. and Levine, A. (2004) Rapid transmission of oxidative and nitrosative stress signals from roots to shoots in Arabidopsis. *Plant Physiol Biochem*, **42**, 425–428.

Cobbett, C.S., May, M.J., Howden, R. and Rolls, B. (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. *Plant J*, **16**, 73–78.

Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J. and Vangronsveld, J. (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J Plant Physiol*, **168**, 309–316.

Cuypers, A., Keunen, E., Bohler, S., Jozefczak, M., Opdenakker, K., Gielen, H., Vercampt, H., Bielen, A., Schellingen, K., Vangronsveld, J. and Remans, T. (2012) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling, in: Gupta, D.K., Sandalio, L.M. (Eds.), Metal toxicity in plants: Perception, signaling and remediation. Springer-Verlag GmbH, Berlin, Heidelberg, pp. 65–90.

Cvetkovska, M. and Vanlerberghe, G.C. (2012) Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol*, **195**, 32–39.

DalCorso, G., Farinati, S., Maistri, S. and Furini, A. (2008) How plants cope with cadmium: Staking all on metabolism and gene expression. *J Integr Plant Biol*, **50**, 1268–1280.

Dupae, J., Bohler, S., Noben, J.P., Carpentier, S., Vangronsveld, J. and Cuypers, A. (2014) Problems inherent to a meta-analysis of proteomics data: A case study on the plants' response to Cd in different cultivation conditions. *J Proteomics*, **108**, 30–54.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R. and Van Breusegem, F. (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. *Plant Physiol*, **141**, 436–445.

Galston, A.W. and Sawhney, R.K. (1990) Polyamines in plant physiology. *Plant Physiol*, **94**, 406–410.

Geuns, J.M.C., Cuypers, A.J.F., Michiels, T., Colpaert, J.V., Van Laere, A., Van Den Broeck, K.A.O. and Vandecasteele, C.H.A. (1997) Mung bean seedlings as bio-indicators for soil and water contamination by cadmium. *Sci Total Environ*, **203**, 183–197.

Gill, S.S. and Tuteja, N. (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signal Beh*, **5**, 26–33.

Groppa, M.D., Benavides, M.P. and Tomaro, M.L. (2003) Polyamine metabolism in sunflower and wheat leaf discs under cadmium or copper stress. *Plant Sci*, **164**, 293–299.

Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L.M., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J.P., Vavasseur, A. and Leonhardt, N. (2006) Genome-wide transcriptome profiling of the early cadmium response of *Arabidopsis* roots and shoots. *Biochimie*, **88**, 1751–1765.

Heyno, E., Klose, C. and Krieger-Liszkay, A. (2008) Origin of cadmium-induced reactive oxygen species production: mitochondrial electron transfer versus plasma membrane NADPH oxidase. *New Phytol*, **179**, 687–699.

Imberty, A., Goldberg, R. and Catesson, A.M. (1984) Tetramethylbenzidine and *p*-phenylenediamine-pyrocatechol for peroxidase histochemistry and biochemistry: Two new, non-carcinogenic chromogens for investigating lignification process. *Plant Sci Lett*, **35**, 103–108.

Jozefczak, M., Keunen, E., Schat, H., Bliek, M., Hernández, L.E., Carleer, R., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2014) Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. *Plant Physiol Biochem*, **83**, 1–9.

Keunen, E., Truyens, S., Bruckers, L., Remans, T., Vangronsveld, J. and Cuypers, A. (2011) Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged? *Plant Physiol Biochem*, **49**, 1084–1091.

Keunen, E., Jozefczak, M., Remans, T., Vangronsveld, J. and Cuypers, A. (2013a) Alternative respiration as a primary defence during cadmium-induced mitochondrial oxidative challenge in *Arabidopsis thaliana*. *Environ Exp Bot*, **91**, 63–73.

Keunen, E., Remans, T., Opdenakker, K., Jozefczak, M., Gielen, H., Guisez, Y., Vangronsveld, J. and Cuypers, A. (2013b) A mutant of the *Arabidopsis thaliana* LIPOXYGENASE1 gene shows altered signalling and oxidative stress related responses after cadmium exposure. *Plant Physiol Biochem*, **63**, 272–280.

Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmüller, E., Dörmann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A.R. and Steinhauser, D. (2005) GMD@CSB.DB: the Golm metabolome database. *Bioinformatics*, **21**, 1635–1638.

Lehmann, M., Schwarzländer, M., Obata, T., Sirikantaramas, S., Burow, M., Olsen, C.E., Tohge, T., Fricker, M.D., Møller, B.L., Fernie, A.R., Sweetlove, L.J. and Laxa, M. (2009) The metabolic response of *Arabidopsis* roots to oxidative stress is distinct from that of heterotrophic cells in culture and highlights a complex relationship between the levels of transcripts, metabolites, and flux. *Mol Plant*, **2**, 390–406.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L. and Fernie, A.R. (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat Protoc*, **1**, 387–396.

Lunn, J.E., Delorge, I., Figueroa, C.M., Van Dijck, P. and Stitt, M. (2014) Trehalose metabolism in plants. *Plant J*, **79**, 544–567.

McCord, J.M. and Fridovich, I. (1969) Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). *J Biol Chem*, **244**, 6049–6055.

Niittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A.M. and Zeeman, S.C. (2004) A previously unknown maltose transporter essential for starch degradation in leaves. *Science*, **303**, 87–89.

Nishizawa, A., Yabuta, Y. and Shigeoka, S. (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiol*, **147**, 1251–1263.

Obata, T. and Fernie, A.R. (2012) The use of metabolomics to dissect plant responses to abiotic stresses. *Cell Mol Life Sci*, **69**, 3225–3243.

Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J. and Mauch F. (2006) Identification of *PAD2* as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. *Plant J*, **49**, 159–172.

R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rauser, W.E. (2001) The role of glutathione in plant reaction and adaptation to excess metals, in: Grill, D., Tausz, M., Kok, L.J. (Eds.), Significance of glutathione to plant adaptation to the environment. Kluwer, Dordrecht, pp. 123–154.

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J. and Cuypers, A. (2008) Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta*, **227**, 1343–1349.

Sandalio, L.M., Dalurzo, H.C., Gómez, M., Romero-Puertas, M.C. and del Río, L.A. (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J Exp Bot*, **52**, 2115–2126.

Sarry, J.E., Kuhn, L., Ducruix, C., Lafaye, A., Junot, C., Hugouvieux, V., Jourdain, A., Bastien, O., Fievet, J.B., Vailhen, D., Amekraz, B., Moulin, C., Ezan, E., Garin, J. and Bourguignon, J. (2006) The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. *Proteomics*, **6**, 2180–2198.

Schauer, N., Steinhauser, D., Strelkov, S., Schomburg, D., Allison, G., Moritz, T., Lundgren, K., Roessner-Tunali, U., Forbes, M.G., Willmitzer, L., Fernie, A.R. and Kopka, J. (2005) GC–MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Lett*, **579**, 1332–1337.

Schellingen, K., Van Der Straeten, D., Vandenbussche, F., Prinsen, E., Remans, T., Vangronsveld, J. and Cuypers, A. (2014) Cadmium-induced ethylene production and responses in *Arabidopsis thaliana* rely on *ACS2* and *ACS6* gene expression. *BMC Plant Biol*, **14**, 214.

Semane, B., Dupae, J., Cuypers, A., Noben, J.P., Tuomainen, M., Tervahauta, A., Kärenlampi, S., Van Belleghem, F., Smeets, K. and Vangronsveld, J. (2010) Leaf proteome responses of *Arabidopsis thaliana* exposed to mild cadmium stress. *J Plant Physiol*, **167**, 247–254.

Seth, C.S., Remans, T., Keunen, E., Jozefczak, M., Gielen, H., Opdenakker, K., Weyens, N., Vangronsveld, J. and Cuypers, A. (2012) Phytoextraction of toxic metals: a central role for glutathione. *Plant Cell Environ*, **35**, 334–346.

Sharma, S.S. and Dietz, K.J. (2006) The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot*, **57**, 711–726.

Smeets, K., Ruytinx, J., Van Belleghem, F., Semane, B., Lin, D., Vangronsveld, J. and Cuypers, A. (2008a) Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. *Plant Physiol Biochem*, **46**, 212–218.

Smeets, K., Ruytinx, J., Semane, B., Van Belleghem, F., Remans, T., Van Sanden, S., Vangronsveld, J. and Cuypers, A. (2008b) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environ Exp Bot*, **63**, 1–8.

Sun, X., Zhang, J., Zhang, H., Ni. Y., Zhang, Q., Chen, J. and Guan, Y. (2010) The responses of *Arabidopsis thaliana* to cadmium exposure explored via metabolite profiling. *Chemosphere*, **78**, 840–845.

Van Belleghem, F. (2007) Cadmium responses in *Arabidopsis thaliana*: a study focusing on subcellular localization, effects on the cellular ultrastructure and photosynthesis in relation to oxidative stress. PhD Thesis. Hasselt University: Belgium.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*, **3**, research0034.1–034.11.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D. and Mench, M. (2009) Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ Sci Pollut Res*, **16**, 765–794.

Vanlerberghe, G.C., Cvetkovska, M. and Wang, J. (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? *Physiol Plant*, **137**, 392–406.

Vanlerberghe, G.C. (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci*, **14**, 6805-6847.

Watanabe, C.K., Hachiya, T., Terashima, I. and Noguchi, K. (2008) The lack of alternative oxidase at low temperature leads to a disruption of the balance in carbon and nitrogen metabolism, and to an up-regulation of antioxidant defence systems in *Arabidopsis thaliana* leaves. *Plant Cell Environ*, **31**, 1190–1202.

Weise, S.E., Weber, A.P.M. and Sharkey, T.D. (2004) Maltose is the major form of carbon exported from the chloroplast at night. *Planta*, **218**, 474–482.

SUPPLEMENTARY INFORMATION

Supplementary Table 6.1. *Forward (FW) and reverse (REV) primers used to determine gene expression levels via quantitative real-time PCR. E-E-jn, exon-exon junction; UTR, untranslated region; FBOX, F-box protein; PPR, pentatricopeptide repeat; SAND, SAND family; TIP41-like, tonoplast intrinsic protein 41-like; UPOX, upregulated by oxidative stress; TIR, Toll-Interleukin-1; LOX, lipoxygenase; RBOH, respiratory burst oxidase homologue; APX, ascorbate peroxidase; CAT, catalase; CSD, Cu/Zn superoxide dismutase; FSD, Fe superoxide dismutase; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; Prx, peroxiredoxin; AOX, alternative oxidase.*

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Reference genes					
AT5G15710	*FBOX protein*	FW: TTTCGGCTGAGAGGTTCGAGT REV: GATTCCAAGACGTAAAGCAGATCAA	Exon 1	63	87.18%
AT5G55840	*PPR gene*	FW: AAGACAGTGAAGGTGCAACCTTACT REV: AGTTTTTGAGTTGTATTTGTCAGAGAAAG	3'-UTR	59	85.90%
AT2G28390	*SAND family*	FW: AACTCTATGCAGCATTTGATCCACT REV: TGATTGCATATCTTTATCGCCATC	Exon 13 and 14	61	107.80%
AT4G34270	*TIP41-like*	FW: GTGAAAACTGTTGGAGAGAAGCAA REV: TCAACTGGATACCCTTTCGCA	E1-E2-jn and exon 2	61	88.65%
Genes encoding markers for ROS-induced gene expression					
AT2G21640	*UPOX*	FW: GACTTGTTTCAAAAACACCATGGAC REV: CACTTCCTTAGCCTCAATTTGCTTC	Exon 1 and 2	91	93.79%
AT2G43510	*Defensin-like*	FW: ATGGCAAAGGCTATCGTTTCC REV: CGTTACCTTGCGCTTCTATCTCC	Exon 1 and 2	91	97.81%
AT1G19020	*Unknown*	FW: GAAAATGGGACAAGGGTTAGACAAA REV: CCCAACGAAAACCAATAGCAGA	Exon 1	92	96.56%
AT1G05340	*Unknown*	FW: TCGGTAGCTCAGGGTAAAGTGG REV: CCAGGGCACAACAGCAACA	Exon 2 and 3	91	98.20%
AT1G57630	*TIR-class*	FW: ACTCAAACAGGCGATCAAAGGA REV: CACCAATTCGTCAAGACAACACC	Exon 1	91	101.52%
Genes encoding ROS producing enzymes					
AT1G55020	*LOX1*	FW: TTGGCTAAGGCTTTTGTCGG REV: GTGGCAATCACAAACGGTTC	Exon 6 and 7	101	94.40%
AT1G72520	*LOX4*	FW: AAGGTCTCCCTGCTGATCTCAT REV: AAGCCCATGTGGTTGTGTTG	Exon 5 and 6	68	95.71%
AT3G22400	*LOX5*	FW: GGCAAAACCGGCCGTAAAT REV: CGTCCCTTGGCACGTATATGTT	Exon 3 and 4	91	101.96%
AT1G67560	*LOX6*	FW: GGCGATTTGACATGGAAGGA REV: ACAAGCCTCACGCCACATTC	Exon 8 and 9	91	108.00%

AGI	Annotation	Primer sequences (5'-3')	Exon location	Amplicon size (bp)	Primer efficiency
Genes encoding ROS producing enzymes					
AT5G51060	*RBOHC*	FW: TCACCAGAGACTGGCACAATAAA REV: GATGCTCGACCTGAATGCTC	Exon 6 and 7	101	92.31%
AT5G47910	*RBOHD*	FW: AACTCTCCGCTGATTCCAACG REV: TGGTCAGCGAAGTCTTTAGATTCCT	Exon 1	91	93.96%
AT1G64060	*RBOHF*	FW: GGTGTCATGAACGAAGTTGCA REV: AATGAGAGCAGAACGAGCATCA	Exon 11 and 12	99	96.58%
Genes encoding antioxidative enzymes					
AT1G07890	*APX1*	FW: TGCCACAAGGATAGGTCTGG REV: CCTTCCTTCTCTCCGCTCAA	Exon 5 and 6	101	89.24%
AT1G20630	*CAT1*	FW: AAGTGCTTCATCGGGAAGGA REV: CTTCAACAAAACGCTTCACGA	E5-E6-jn and exon 7	103	101.89%
AT4G35090	*CAT2*	FW: AACTCCTCCATGACCGTTGGA REV: TCCGTTCCCTGTCGAAATTG	Exon 2 and 3	76	99.26%
AT1G20620	*CAT3*	FW: TCTCCAACAACATCTCTTCCCTCA REV: GTGAAATTAGCAACCTTCTCGATCA	Exon 2 and 3	91	95.80%
AT1G08830	*CSD1*	FW: TCCATGCAGACCCTGATGAC REV: CCTGGAGACCAATGATGCC	Exon 5 and E6-E7-jn	102	87.52%
AT2G28190	*CSD2*	FW: GAGCCTTTGTGGTTCACGAG REV: CACACCACATGCCAATCTCC	Exon 6 and E7-E8-jn	101	98.56%
AT4G25100	*FSD1*	FW: CTCCCAATGCTGTGAATCCC REV: TGGTCTTCGGTTCTGGAAGTC	Exon 4 and E6-E7-jn	101	85.58%
AT3G24170	*GR1*	FW: CTCAAGTGTGGAGCAACCAAAG REV: ATGCGTCTGGTCACACTGC	Exon 15 and 16	101	98.61%
Genes encoding mitochondrial antioxidative enzymes					
AT4G08390	*APX*	FW: GGACACCAGAGTGGCTGAAGTT REV: GCATCAGTGGGTAGGACAAGGAG	Exon 7 and 8	91	96.26%
AT3G54660	*GR2*	FW: TAGGGTTGGAGAATGTTGGCG REV: GCCCAGATGGATGGAACAGAT	Exon 5 and 6	91	101.52%
AT1G63940	*MDHAR*	FW: TAGTAGAAAGCGGATCGCCTGA REV: GCGCTTGCGAGTTTAGCCTT	Exon 16 and 17	91	101.88%
AT3G06050	*Prx*	FW: GCTATCAATGGTTGGGCAGAGA REV: GCCCCAAGCTTTTGTGAAATT	Exon 3 and 4	91	95.94%
Genes encoding alternative oxidases (AOX)					
AT3G22370	*AOX1a*	FW: CTCTTCGTTGGCCTACCGATT REV: AACCATTCCAGGTACTGCTGCTAC	Exon 2 and 3	92	89.73%

Supplementary Table 6.2. *Summary of the reference genes used in each gene expression experiment. AOX, alternative oxidase; FBOX, F-box protein; GSH, glutathione; PPR, pentatricopeptide repeat; SAND, SAND family; TIP41-like, tonoplast intrinsic protein 41-like.*

Experiment	AGI	Annotation
Wild-type and *aox1a* knockout plants	*AT5G15710*	*FBOX protein*
	AT5G55840	*PPR gene*
	AT4G34270	*TIP41-like*
Wild-type and GSH-deficient plants	*AT5G55840*	*PPR gene*
	AT2G28390	*SAND family*
	AT4G34270	*TIP41-like*

Supplementary Table 6.3. *Quantitative real-time PCR parameters according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines derived from Bustin et al. (2009). * All procedures were performed according to the manufacturer's protocols.*

Sample/Template	
Source	Roots of *Arabidopsis thaliana* plants cultivated in hydroponics
Method of preservation	Liquid nitrogen
Storage time	3 weeks at - 70 °C
Handling	Frozen
Extraction method	Phenol-free Total RNA isolation: RNAqueous® Total RNA Isolation Kit* (Ambion, Life Technologies, Belgium)
RNA: DNA-free	TURBO DNA-*free*™ Kit* (Ambion, Life Technologies, Belgium) Design of intron-spanning primers whenever possible
Concentration	NanoDrop®: ND-1000 Spectrophotometer (ThermoScientific, USA)
Assay optimisation and validation	
Accession number	Supplementary Table 6.1
Amplicon details	Exon location and amplicon size: Supplementary Table 6.1
Primer sequences	Supplementary Table 6.1
In silico	Primers were blasted using the BLAST tool at http://arabidopsis.org/
Empirical	A primer concentration of 300 nM was used Annealing temperature: 60 °C
Priming conditions	Combination of oligodT-primers and random hexamers
PCR efficiency	Dilution series (slope, y-intercept and r^2; Supplementary Table 6.1)
Linear dynamic range	Samples are situated within the range of the efficiency curve
Reverse transcription - PCR	
Protocols	As stated in the Materials and Methods section
Reagents	As stated in the Materials and Methods section
No template control (NTC)	Cq and dissociation curve verification
Data analysis	
Specialist software	7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Belgium) Software v2.0.1
Statistical justification	4 biological replicates Outliers were eliminated after statistical validation using the extreme studentised deviate analysis (GraphPad Software, Inc.) at significance level 0.05 Log transformation of the data Two-way ANOVA and the Tukey-Kramer post-hoc test to correct for multiple comparisons using R version 2.13.1
Normalisation	3 reference genes were selected (Supplementary Table 6.2)

Supplementary Table 6.4. *Overview of the metabolite reporting list for roots (A) and leaves (B).*

A (roots)

Experiment title:		Metabolite changes in Cd-exposed WT versus *aox1a* knockout plants					
Organism/plant species:		*Arabidopsis thaliana*					
Organ/tissue:		root					
Analytical tool:		GC-TOF-MS					
Peak/compound no.- number referenced back to the main text							
Ret. Time - Tag Time Index and Time deviation							
Putative Name - putative identification of the metabolite/derivative							
Corresponding metabolite name in literature							
Mol. Formula - molecular formula of the metabolite or its FA adduct							
Mass to charge ratio (m/z)							
Peak/compound no.	**Tag Time Index**	**Time Deviation**	**Putative metabolite name (Derivative)**	**Corresponding metabolite in literature**	**Metabolite Class**	**Mol. formula**	**Mass to charge ratio (m/z)**
1	223152	0.23	M000071_A104002-101_METB_222650_TOF_Pyruvic acid (1MEOX) (1TMS)	Pyruvate	Organic acid	C3H4O3	174
2	271529	0	M000030_A122001-101_METB_271580_TOF_Valine, DL- (2TMS)	Valine	Amino acid	C5H11NO2	144
3	318957	-0.03	M000017_A132002-101_METB_319193_TOF_Isoleucine, L- (2TMS)	Isoleucine	Amino acid	C6H13NO2	158
4	324960	-0.06	M000031_A133001-101_METB_325180_TOF_Glycine (3TMS)	Glycine	Amino acid	C2H5NO2	174
5	338465	0.38	M000029_A132003-101_METB_338693_TOF_Proline, L- (2TMS)	Proline	Amino acid	C5H9NO2	142
6	333246	-0.08	M000075_A129001-101_METB_333520_TOF_Phosphoric acid (3TMS)	Phosphoric acid	Mineral inorganic acid	H3PO4	283
7	344753	-0.08	M000073_A135003-101_METB_345050_TOF_Glyceric acid, DL- (3TMS)	Glycerate	Organic acid	C3H6O4	133
8	353911	-0.05	M000026_A138002-101_METB_354060_TOF_Alanine, DL- (3TMS)	Alanine	Amino acid	C3H7NO2	188
9	357477	0.02	M000015_A138001-101_METB_357523_TOF_Serine, DL- (3TMS)	Serine	Amino acid	C3H7NO3	204
10	365310	-0.06	M000074_A134001-101_METB_365427_TOF_Succinic acid (2TMS)	Succinate	Organic acid	C4H6O4	129
11	371272	-0.05	M000067_A137001-101_METB_371255_TOF_Fumaric acid (2TMS)	Fumarate	Organic acid	C4H4O4	245
12	394136	-0.03	M000027_A144001-101_METB_394250_TOF_Alanine, beta- (3TMS)	β-alanine	Amino acid (non-protein)	C3H7NO2	174
13	441410	0.1	M000065_A149001-101_METB_440995_TOF_Malic acid, DL- (3TMS)	Malate	Organic acid	C4H6O5	217
14	452250	0.02	M000114_A153003-101_METB_452200_TOF_Butyric acid, 4-amino- (3TMS)	GABA	Amino acid (non-protein)	C4H9NO2	174
15	457484	0.07	M000033_A152002-101_METB_457283_TOF_Aspartic acid, L- (3TMS)	Aspartate	Amino acid	C4H7NO4	232
16	474181	-0.04	M000018_A152001-101_METB_474325_TOF_Methionine, DL- (2TMS)	Methionine	Amino acid	C5H11NO2S	128
17	492519	-0.01	M000032_A174008-101_MST_492650_TOF_Glutamine, DL- (4TMS)	Glutamine	Amino acid	C5H10N2O3	227
18	507990	-0.09	M000036_A163001-101_METB_507780_TOF_Glutamic acid, DL- (3TMS)	Glutamate	Amino acid	C5H9NO5	128
19	517057	-0.02	M000186_A175002-101_METB-METB_517180_TOF_Putrescine (4TMS)	Putrescine	Polyamine	C4H12N2	174
20	523816	0.01	M000571_A158004-101_METB_523847_TOF_Glutaric acid, 2-oxo- (1MEOX) (2TMS)	2-oxoglutarate	Organic acid	C5H6O5	198
21	530705	-0.12	M000011_A164001-101_METB_531145_TOF_Phenylalanine, DL- (2TMS)	Phenylalanine	Amino acid	C9H11NO2	192
22	550153	0.03	M000013_A168001-101_METB_550100_TOF_Asparagine, DL- (3TMS)	Asparagine	Amino acid	C4H8N2O3	116
23	570352	0	M000028_A182002-101_METB-METB_570427_TOF_Ornithine, DL- (4TMS)	Ornithine	Amino acid	C5H12N2O2	142
24	573984	-0.06	M000328_A177002-101_METB_574230_TOF_Glycerol-3-phosphate, DL- (4TMS)	Glycerol-3-phosphate	Phosphate	C3H9O6P	299
25	579134	-0.04	M000606_A187002-101_METB_579380_TOF_Fructose, D- (1MEOX) (5TMS)	Fructose	Sugar (hexose)	C6H12O6	217
26	590387	0	M000040_A189002-101_METB_590400_TOF_Glucose, D- (1MEOX) (5TMS)	Glucose	Sugar (hexose)	C6H12O6	160
27	592584	-0.04	M000069_A182004-101_METB-METB_592883_TOF_Citric acid (4TMS)	Citrate	Organic acid	C6H8O7	273
28	615122	-0.06	M000014_A192003-101_METB_615467_TOF_Lysine, L- (4TMS)	Lysine	Amino acid	C6H14N2O2	156
29	624686	0.1	M000082_A185002-101_METB-METB_624990_TOF_Dehydroascorbic acid dimer (TMS)	Dehydroascorbate	Hydroxy acid	C6H6O6	157
30	650900	-0.17	M000001_A195002-101_METB_651990_TOF_Ascorbic acid, L(+)- (4TMS)	Ascorbate	Organic acid	C6H8O6	117
31	654140	0.04	M000060_A209002-101_METB_653910_TOF_Inositol, myo- (6TMS)	Inositol	Polyol	C6H14O6	191
32	789778	-0.07	M000012_A223001-101_METB_790560_TOF_Tryptophan, L- (3TMS)	Tryptophan	Amino acid	C11H12N2O2	202
33	840470	-0.03	M000044_A264001-101_METB_840783_TOF_Sucrose, D- (8TMS)	Sucrose	Sugar (disaccharide)	C12H22O11	361
34	875334	-0.1	M000671_A274002-101_METB_876240_TOF_Trehalose, alpha,alpha'-, D- (8TMS)	Trehalose	Sugar (disaccharide)	C12H22O11	191
35	1032950	-0.04	M000049_A337002-101_METB_1033367_TOF_Raffinose (11TMS)	Raffinose	Sugar (trisaccharide)	C18H32O16	217

Supplementary Table 6.4. *Continued.*

B (leaves)

Experiment title:		Metabolite changes in Cd-exposed WT versus *aox1a* knockout plants					
Organism/plant species:		*Arabidopsis thaliana*					
Organ/tissue:		leaf					
Analytical tool:		GC-TOF-MS					
Peak/compound no.- number referenced back to the main text							
Ret. Time - Tag Time Index and Time deviation							
Putative Name - putative identification of the metabolite/derivative							
Corresponding metabolite name in literature							
Mol. Formula - molecular formula of the metabolite or its FA adduct							
Mass to charge ratio (m/z)							
Peak/compound no.	**Tag Time Index**	**Time Deviation**	**Putative metabolite name (Derivative)**	**Corresponding metabolite in literature**	**Metabolite Class**	**Mol. formula**	**Mass to charge ratio (m/z)**
1	223207	-0.28	M000071_A104002-101_METB_222650_TOF_Pyruvic acid (1MEOX) (1TMS)	Pyruvate	Organic acid	C3H4O3	174
2	271523	-0.01	M000030_A122001-101_METB_271580_TOF_Valine, DL- (2TMS)	Valine	Amino acid	C5H11NO2	144
3	318946	-0.02	M000017_A132002-101_METB_319193_TOF_Isoleucine, L- (2TMS)	Isoleucine	Amino acid	C6H13NO2	158
4	324980	-0.06	M000031_A133001-101_METB_325180_TOF_Glycine (3TMS)	Glycine	Amino acid	C2H5NO2	86
5	338410	-0.09	M000029_A132003-101_METB_338693_TOF_Proline, L- (2TMS)	Proline	Amino acid	C5H9NO2	142
6	340448	0.01	M000364_A127002-101_METB_340357_TOF_Urea (2TMS)	Urea	Organic nitrogen compound	CH4N2O	171
7	353803	-0.07	M000026_A138002-101_METB_354060_TOF_Alanine, DL- (3TMS)	Alanine	Amino acid	C3H7NO2	100
8	357543	0.02	M000015_A138001-101_METB_357523_TOF_Serine, DL- (3TMS)	Serine	Amino acid	C3H7NO3	204
9	365292	-0.05	M000074_A134001-101_METB_365427_TOF_Succinic acid (2TMS)	Succinate	Organic acid	C4H6O4	129
10	367584	-0.04	M000016_A140001-101_METB_368365_TOF_Threonine, DL- (3TMS)	Threonine	Amino acid	C4H9NO3	117
11	371768	0.17	M000067_A137001-101_METB_371255_TOF_Fumaric acid (2TMS)	Fumarate	Organic acid	C4H4O4	143
12	394007	-0.05	M000027_A144001-101_METB_394250_TOF_Alanine, beta- (3TMS)	β-alanine	Amino acid (non-protein)	C3H7NO2	100
13	441204	0.23	M000065_A149001-101_METB_440995_TOF_Malic acid, DL- (3TMS)	Malate	Organic acid	C4H6O5	233
14	452153	0.19	M000114_A153003-101_METB_452200_TOF_Butyric acid, 4-amino- (3TMS)	GABA	Amino acid (non-protein)	C4H9NO2	174
15	457370	0.17	M000033_A152002-101_METB_457283_TOF_Aspartic acid, L- (3TMS)	Aspartate	Amino acid	C4H7NO4	232
16	458331	-0.06	M000078_A156001-101_METB_458330_TOF_Threonic acid (4TMS)	Threonate	Hydroxy acid	C4H8O5	117
17	474181	-0.1	M000018_A152001-101_METB_474325_TOF_Methionine, DL- (2TMS)	Methionine	Amino acid	C5H11NO2S	128
18	490705	-0.04	M000028_A162001-101_METB-METB_490485_TOF_Ornithine, DL- (3TMS)	Ornithine	Amino acid	C5H12N2O2	142
19	492511	-0.02	M000032_A174008-101_MST_492650_TOF_Glutamine, DL- (4TMS)	Glutamine	Amino acid	C5H10N2O3	227
20	507945	-0.08	M000036_A163001-101_METB_507780_TOF_Glutamic acid, DL- (3TMS)	Glutamate	Amino acid	C5H9NO5	128
21	517071	-0.01	M000186_A175002-101_METB-METB_517180_TOF_Putrescine (4TMS)	Putrescine	Polyamine	C4H12N2	174
22	530715	-0.12	M000011_A164001-101_METB_531145_TOF_Phenylalanine, DL- (2TMS)	Phenylalanine	Amino acid	C9H11NO2	192
23	550128	0.01	M000013_A168001-101_METB_550100_TOF_Asparagine, DL- (3TMS)	Asparagine	Amino acid	C4H8N2O3	116
24	579260	0	M000606_A187002-101_METB_579380_TOF_Fructose, D- (1MEOX) (5TMS)	Fructose	Sugar (hexose)	C6H12O6	217
25	592622	0.19	M000069_A182004-101_METB-METB_592883_TOF_Citric acid (4TMS)	Citrate	Organic acid	C6H8O7	273
26	598751	-0.03	M000040_A191001-101_METB-METB_598880_TOF_Glucose, D- (1MEOX) (5TMS)	Glucose	Sugar (hexose)	C6H12O6	319
27	615109	-0.07	M000014_A192003-101_METB_615467_TOF_Lysine, L- (4TMS)	Lysine	Amino acid	C6H14N2O2	156
28	654251	0.06	M000060_A209002-101_METB_653910_TOF_Inositol, myo- (6TMS)	Inositol	Polyol	C6H14O6	191
29	676779	-0.25	M000700_A214008-101_METB_679350_TOF_Glucoheptose (1MEOX) (6TMS)	Glucoheptose	Sugar (heptose/aldose)	C7H14O7	217
30	723437	0.15	M000106_A226002-101_METB_723585_TOF_Spermidine (5TMS)	Spermidine	Polyamine	C7H19N3	144
31	814628	-0.11	M000012_A219006-101_METB_815200_TOF_Tryptophan, L- (2TMS)	Tryptophan	Amino acid	C11H12N2O2	130
32	869442	-0.1	M000048_A274001-101_METB_870355_TOF_Maltose, D- (1MEOX) (8TMS)	Maltose	Sugar (hexose)	C12H22O11	204
33	875360	-0.1	M000671_A274002-101_METB_876240_TOF_Trehalose, alpha,alpha'-, D- (8TMS)	Trehalose	Sugar (disaccharide)	C12H22O11	191
34	1033057	-0.03	M000049_A337002-101_METB_1033367_TOF_Raffinose (11TMS)	Raffinose	Sugar (trisaccharide)	C18H32O16	204

Supplementary Table 6.5. *Metabolic profiling in roots of wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants. (A) Time-dependent changes (0, 24 and 72 h) in metabolite levels compared for unexposed WT and aox1a knockout plants. Data are given as the mean ± S.E. of 6 biological replicates relative to the unexposed WT at 0 h set at 1.00. Different letters represent significant differences within and between both genotypes ($p < 0.05$) (two-way ANOVA). (B) Metabolite levels measured in roots of WT versus aox1a knockout plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 6 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced abundance changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for higher and lower levels respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$) (two-way ANOVA per time point).*

Supplementary Table 6.5. Part A.

A. Amino acids						
	0 h		**24 h**		**72 h**	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Alanine	1.00 ± 0.13a	0.97 ± 0.16a	1.12 ± 0.23a	1.07 ± 0.12a	0.85 ± 0.14a	0.69 ± 0.06a
β-alanine	1.00 ± 0.05a,b	1.09 ± 0.07a	1.02 ± 0.09a,c	1.07 ± 0.04a	0.72 ± 0.04b	0.80 ± 0.05b,c
Asparagine	1.00 ± 0.30a	0.92 ± 0.27a	0.74 ± 0.15a	0.73 ± 0.17a	0.59 ± 0.12a	0.73 ± 0.25a
Aspartate	1.00 ± 0.07a,b	1.05 ± 0.03a	0.75 ± 0.09b,c,d	0.76 ± 0.05c	0.60 ± 0.02d	0.61 ± 0.03c,d
GABA	1.00 ± 0.07a,c	0.45 ± 0.02b	1.29 ± 0.17a	1.11 ± 0.12a,c	0.90 ± 0.05a,c	0.80 ± 0.07c
Glutamate	1.00 ± 0.14a	1.07 ± 0.08a	1.30 ± 0.10a	1.40 ± 0.10a	0.92 ± 0.11a	1.08 ± 0.09a
Glutamine	1.00 ± 0.09a	0.84 ± 0.09a	0.61 ± 0.09a,c	0.68 ± 0.16a,c	0.24 ± 0.03b	0.22 ± 0.04b,c
Glycine	1.00 ± 0.11a	1.05 ± 0.02a	0.37 ± 0.04b	0.39 ± 0.01b	0.22 ± 0.01c	0.21 ± 0.01c
Isoleucine	1.00 ± 0.08a	0.91 ± 0.03a	0.46 ± 0.03b	0.44 ± 0.02b	0.52 ± 0.02b	0.51 ± 0.03b
Lysine	1.00 ± 0.09a	0.89 ± 0.03a	0.54 ± 0.04b	0.58 ± 0.04b	0.58 ± 0.04b	0.62 ± 0.08b
Methionine	1.00 ± 0.06a	0.89 ± 0.01a	1.07 ± 0.10a	0.99 ± 0.03a	0.84 ± 0.02a	0.89 ± 0.05a
Ornithine	1.00 ± 0.14a	0.81 ± 0.02a	0.15 ± 0.00b	0.16 ± 0.00b	0.13 ± 0.00c	0.12 ± 0.01c
Phenyl-alanine	1.00 ± 0.09a	0.88 ± 0.03a	0.77 ± 0.04a	0.79 ± 0.04a	0.81 ± 0.03a	0.84 ± 0.06a
Proline	1.00 ± 0.13a,b	1.00 ± 0.05a	0.68 ± 0.05b,d	0.79 ± 0.09a,b	0.47 ± 0.01c	0.51 ± 0.03c,d
Serine	1.00 ± 0.10a,b	0.99 ± 0.02a	0.51 ± 0.03b	0.52 ± 0.02b	0.41 ± 0.01b,c	0.39 ± 0.02c
Tryptophan	1.00 ± 0.09a	1.01 ± 0.07a	1.00 ± 0.09a	1.11 ± 0.08a	0.95 ± 0.10a	0.98 ± 0.06a
Valine	1.00 ± 0.06a	0.95 ± 0.01a	0.59 ± 0.04b	0.61 ± 0.03b	0.68 ± 0.04b	0.68 ± 0.04b

Supplementary Table 6.5. Part A. *Continued.*

B. Organic acids

	0 h		24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Ascorbate	1.00 ± 0.15a	1.18 ± 0.13a	0.56 ± 0.24a	0.74 ± 0.33a	0.53 ± 0.16a	0.87 ± 0.26a
Citrate	1.00 ± 0.10a	0.92 ± 0.07a	1.30 ± 0.10a,b	1.06 ± 0.06a	1.61 ± 0.15b,c	1.95 ± 0.19c
Dehydro-ascorbate	1.00 ± 0.12a	1.07 ± 0.16a	0.80 ± 0.13a	0.80 ± 0.13a	0.65 ± 0.10a	0.83 ± 0.12a
Fumarate	1.00 ± 0.07a,b	0.99 ± 0.06a,b	1.02 ± 0.03a	0.88 ± 0.02b	0.91 ± 0.02b	0.84 ± 0.06a,b
Glutarate (2-oxo)	1.00 ± 0.07a	0.88 ± 0.03a	1.81 ± 0.18b	1.84 ± 0.14b	2.02 ± 0.12b	2.12 ± 0.13b
Glycerate	1.00 ± 0.11a	0.97 ± 0.03a	2.43 ± 0.57b,c	2.41 ± 0.36b	5.14 ± 0.89c,d	4.28 ± 0.20d
Malate	1.00 ± 0.07a	0.90 ± 0.02a	1.73 ± 0.27b	1.72 ± 0.15b	2.08 ± 0.16b	1.98 ± 0.12b
Pyruvate	1.00 ± 0.10a	1.45 ± 0.04b	0.78 ± 0.04a	0.80 ± 0.04a	0.76 ± 0.05a	0.75 ± 0.06a
Succinate	1.00 ± 0.06a	0.88 ± 0.05a	1.56 ± 0.17b	1.53 ± 0.13b	1.66 ± 0.04b	1.69 ± 0.07b

C. Sugars

	0 h		24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Fructose	1.00 ± 0.08a	0.83 ± 0.02a	2.06 ± 0.19b	2.21 ± 0.15b	0.99 ± 0.16a	1.07 ± 0.11a
Glucose	1.00 ± 0.07a	0.78 ± 0.06a	1.46 ± 0.18b	1.49 ± 0.08b	0.90 ± 0.04a	0.86 ± 0.09a
Raffinose	1.00 ± 0.05a,b	1.04 ± 0.08a,b	1.39 ± 0.10a	1.43 ± 0.19a	0.86 ± 0.10b	1.01 ± 0.12a,b
Sucrose	1.00 ± 0.06a,c	0.96 ± 0.05a,c	1.15 ± 0.07a,b	1.26 ± 0.07b	0.88 ± 0.06c	0.96 ± 0.05a,c
Trehalose	1.00 ± 0.06a	0.95 ± 0.03a	0.96 ± 0.05a	0.99 ± 0.03a	0.81 ± 0.07a	0.86 ± 0.08a

D. Other metabolites

	0 h		24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Glycerol-3P	1.00 ± 0.09a	0.94 ± 0.05a	0.97 ± 0.05a	0.89 ± 0.03a	0.86 ± 0.04a	0.85 ± 0.05a
Inositol	1.00 ± 0.08a,b	0.92 ± 0.04a	0.96 ± 0.08a,b	1.00 ± 0.06a,b	1.17 ± 0.09a,b	1.25 ± 0.07b
Phosphoric acid	1.00 ± 0.04a,b	0.86 ± 0.06a	1.26 ± 0.09b,c	1.36 ± 0.04c	0.99 ± 0.06a,b	0.94 ± 0.08a
Putrescine	1.00 ± 0.07a	1.00 ± 0.11a,b	0.88 ± 0.11a,b	0.73 ± 0.02b	0.43 ± 0.04c	0.50 ± 0.04c

Supplementary Table 6.5. Part B.

A. Amino acids					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Alanine	**Control**	1.00 ± 0.21	1.00 ± 0.11	1.00 ± 0.17	1.00 ± 0.08
	5 µM Cd	0.86 ± 0.09	1.23 ± 0.16	1.83 ± 0.21	2.85 ± 0.31*
	10 µM Cd	1.40 ± 0.11	1.15 ± 0.13	1.19 ± 0.10	1.62 ± 0.23
β-alanine	**Control**	1.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.06
	5 µM Cd	3.37 ± 0.14	3.30 ± 0.08	2.70 ± 0.09	2.76 ± 0.30
	10 µM Cd	4.43 ± 0.18	4.05 ± 0.14	4.81 ± 0.31	2.88 ± 0.26*
Asparagine	**Control**	1.00 ± 0.21	1.00 ± 0.24	1.00 ± 0.20	1.00 ± 0.35
	5 µM Cd	2.85 ± 0.61	2.44 ± 0.41	9.07 ± 1.53	5.64 ± 1.01
	10 µM Cd	4.76 ± 0.86	5.45 ± 0.91	27.30 ± 4.70	23.20 ± 5.26
Aspartate	**Control**	1.00 ± 0.12	1.00 ± 0.07	1.00 ± 0.04	1.00 ± 0.05
	5 µM Cd	1.80 ± 0.11	2.07 ± 0.20	1.49 ± 0.11	1.59 ± 0.11
	10 µM Cd	2.06 ± 0.08	2.16 ± 0.01	1.99 ± 0.15	2.51 ± 0.10*
GABA	**Control**	1.00 ± 0.13	1.00 ± 0.10	1.00 ± 0.06	1.00 ± 0.09
	5 µM Cd	0.81 ± 0.06	0.96 ± 0.11	1.25 ± 0.07	1.27 ± 0.07
	10 µM Cd	0.92 ± 0.02	1.04 ± 0.10	1.32 ± 0.08	1.10 ± 0.13
Glutamate	**Control**	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.12	1.00 ± 0.09
	5 µM Cd	0.93 ± 0.07	0.98 ± 0.07	1.64 ± 0.16	1.29 ± 0.08
	10 µM Cd	1.25 ± 0.09	1.08 ± 0.02	1.84 ± 0.20	1.84 ± 0.26
Glutamine	**Control**	1.00 ± 0.14	1.00 ± 0.24	1.00 ± 0.13	1.00 ± 0.18
	5 µM Cd	3.25 ± 0.60	3.70 ± 0.67	9.63 ± 1.84	10.07 ± 1.30
	10 µM Cd	6.01 ± 0.58	5.84 ± 0.62	30.78 ± 2.46	82.99 ± 15.33*
Glycine	**Control**	1.00 ± 0.10	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.07
	5 µM Cd	1.01 ± 0.02	1.04 ± 0.14	1.89 ± 0.19	1.62 ± 0.03
	10 µM Cd	1.23 ± 0.02	1.25 ± 0.05	1.61 ± 0.08	1.97 ± 0.06*

Supplementary Table 6.5. Part B. *Continued.*

A. Amino acids (continued)					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Isoleucine	**Control**	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.06
	5 µM Cd	8.54 ± 0.48	7.36 ± 0.96	6.97 ± 0.33	7.28 ± 0.67
	10 µM Cd	20.30 ± 0.55	24.48 ± 1.23	14.80 ± 0.95	12.75 ± 2.48
Lysine	**Control**	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.12
	5 µM Cd	7.45 ± 0.28	6.16 ± 0.59	8.29 ± 0.31	6.70 ± 0.57
	10 µM Cd	13.48 ± 0.52	15.34 ± 0.62	12.13 ± 0.83	9.81 ± 0.28
Methionine	**Control**	1.00 ± 0.09	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.05
	5 µM Cd	1.72 ± 0.06	2.01 ± 0.21	2.93 ± 0.10	2.74 ± 0.22
	10 µM Cd	2.70 ± 0.04	3.56 ± 0.12*	9.53 ± 0.66	8.04 ± 0.76
Ornithine	**Control**	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.08
	5 µM Cd	3.96 ± 0.17	3.27 ± 0.18	7.17 ± 0.17	6.59 ± 0.35
	10 µM Cd	5.39 ± 0.27	5.27 ± 0.27	9.27 ± 0.56	8.99 ± 0.59
Phenylalanine	**Control**	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.07
	5 µM Cd	1.92 ± 0.10	1.65 ± 0.13	1.31 ± 0.10	1.18 ± 0.08
	10 µM Cd	6.61 ± 0.40	6.51 ± 0.28	1.93 ± 0.11	2.11 ± 0.07
Proline	**Control**	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.03	1.00 ± 0.07
	5 µM Cd	1.60 ± 0.11	1.57 ± 0.01	7.87 ± 0.33	7.25 ± 0.53
	10 µM Cd	2.48 ± 0.09	2.25 ± 0.07*	11.10 ± 0.88	8.92 ± 1.15
Serine	**Control**	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.04
	5 µM Cd	1.32 ± 0.05	1.39 ± 0.09	2.63 ± 0.14	2.78 ± 0.13
	10 µM Cd	2.22 ± 0.11	1.94 ± 0.09	4.52 ± 0.23	5.12 ± 0.18
Tryptophan	**Control**	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 011	1.00 ± 0.06
	5 µM Cd	6.54 ± 0.56	5.13 ± 0.46	9.70 ± 0.19	8.45 ± 0.36*
	10 µM Cd	16.61 ± 1.21	14.64 ± 1.01	21.22 ± 1.56	18.73 ± 2.64

Supplementary Table 6.5. Part B. *Continued.*

A. Amino acids (continued)					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Valine	**Control**	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.06
	5 µM Cd	4.96 ± 0.23	4.33 ± 0.46	4.62 ± 0.21	4.65 ± 0.30
	10 µM Cd	10.18 ± 0.32	10.62 ± 0.63	8.67 ± 0.51	7.39 ± 1.02
B. Organic acids					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Ascorbate	**Control**	1.00 ± 0.44	1.00 ± 0.45	1.00 ± 0.30	1.00 ± 0.30
	5 µM Cd	2.67 ± 0.71	4.00 ± 1.18	6.04 ± 0.88	4.60 ± 0.94
	10 µM Cd	3.63 ± 1.10	6.29 ± 0.10	3.13 ± 0.46	4.39 ± 0.81
Citrate	**Control**	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.10
	5 µM Cd	0.54 ± 0.05	0.89 ± 0.10	1.19 ± 0.10	1.04 ± 0.06
	10 µM Cd	1.21 ± 0.16	1.02 ± 0.09	2.08 ± 0.12	1.54 ± 0.13*
Dehydroascorbate	**Control**	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.15	1.00 ± 0.14
	5 µM Cd	1.32 ± 0.03	2.00 ± 0.38	2.20 ± 0.27	2.17 ± 0.31
	10 µM Cd	1.55 ± 0.22	2.18 ± 0.12*	1.73 ± 0.25	1.59 ± 0.20
Fumarate	**Control**	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.07
	5 µM Cd	0.46 ± 0.04	0.41 ± 0.06	0.58 ± 0.03	0.61 ± 0.06
	10 µM Cd	0.56 ± 0.06	0.39 ± 0.04	0.79 ± 0.05	0.49 ± 0.05*
Glutarate (2-oxo)	**Control**	1.00 ± 0.10	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.06
	5 µM Cd	0.25 ± 0.02	0.23 ± 0.02	0.46 ± 0.02	0.48 ± 0.03
	10 µM Cd	0.40 ± 0.01	0.36 ± 0.02	0.40 ± 0.03	0.45 ± 0.06
Glycerate	**Control**	1.00 ± 0.23	1.00 ± 0.15	1.00 ± 0.17	1.00 ± 0.05
	5 µM Cd	0.25 ± 0.03	0.32 ± 0.09	0.11 ± 0.01	0.16 ± 0.03
	10 µM Cd	0.63 ± 0.09	0.64 ± 0.06	0.27 ± 0.01	0.28 ± 0.03

Supplementary Table 6.5. Part B. *Continued.*

B. Organic acids (continued)					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Malate	**Control**	1.00 ± 0.16	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.06
	5 µM Cd	0.17 ± 0.02	0.22 ± 0.05	0.23 ± 0.01	0.26 ± 0.02
	10 µM Cd	0.47 ± 0.05	0.35 ± 0.03	0.60 ± 0.02	0.66 ± 0.09
Pyruvate	**Control**	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.08
	5 µM Cd	0.56 ± 0.06	0.53 ± 0.05	0.65 ± 0.04	0.71 ± 0.06
	10 µM Cd	0.65 ± 0.04	0.58 ± 0.03	0.66 ± 0.02	0.58 ± 0.06
Succinate	**Control**	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.02	1.00 ± 0.04
	5 µM Cd	0.34 ± 0.04	0.34 ± 0.03	0.48 ± 0.02	0.57 ± 0.05
	10 µM Cd	0.42 ± 0.05	0.32 ± 0.03	0.48 ± 0.03	0.47 ± 0.07
C. Sugars					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Fructose	**Control**	1.00 ± 0.09	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.10
	5 µM Cd	0.06 ± 0.00	0.08 ± 0.02	0.31 ± 0.01	0.26 ± 0.02
	10 µM Cd	0.10 ± 0.02	0.05 ± 0.00	0.20 ± 0.02	0.19 ± 0.03
Glucose	**Control**	1.00 ± 0.12	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.10
	5 µM Cd	0.26 ± 0.03	0.26 ± 0.04	0.52 ± 0.02	0.51 ± 0.04
	10 µM Cd	0.61 ± 0.06	0.39 ± 0.03	0.36 ± 0.03	0.38 ± 0.06
Raffinose	**Control**	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.11	1.00 ± 0.12
	5 µM Cd	1.16 ± 0.10	1.27 ± 0.14	4.51 ± 0.14	4.37 ± 0.37
	10 µM Cd	0.94 ± 0.07	1.09 ± 0.06	3.32 ± 0.43	2.37 ± 0.28
Sucrose	**Control**	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.06
	5 µM Cd	0.34 ± 0.03	0.37 ± 0.04	1.54 ± 0.07	1.31 ± 0.08
	10 µM Cd	0.59 ± 0.08	0.28 ± 0.02*	1.89 ± 0.11	1.82 ± 0.25

Supplementary Table 6.5. Part B. *Continued.*

C. Sugars (continued)					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Trehalose	**Control**	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.09	1.00 ± 0.09
	5 µM Cd	1.05 ± 0.08	1.08 ± 0.10	1.42 ± 0.09	1.64 ± 0.13
	10 µM Cd	1.34 ± 0.12	1.18 ± 0.04	1.98 ± 0.20	1.38 ± 0.18*
D. Other metabolites					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Glycerol-3P	**Control**	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.05	1.00 ± 0.06
	5 µM Cd	1.60 ± 0.10	1.70 ± 0.06	3.38 ± 0.19	3.29 ± 0.15
	10 µM Cd	3.10 ± 0.18	3.15 ± 0.14	10.56 ± 0.62	9.41 ± 0.25
Inositol	**Control**	1.00 ± 0.08	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.06
	5 µM Cd	0.97 ± 0.07	1.09 ± 0.11	1.18 ± 0.07	1.38 ± 0.09
	10 µM Cd	1.04 ± 0.09	1.12 ± 0.05	0.47 ± 0.03	0.47 ± 0.07
Phosphoric acid	**Control**	1.00 ± 0.07	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.08
	5 µM Cd	1.46 ± 0.08	1.33 ± 0.08	2.13 ± 0.07	1.98 ± 0.13
	10 µM Cd	1.91 ± 0.05	1.85 ± 0.06	3.00 ± 0.02	2.67 ± 0.13
Putrescine	**Control**	1.00 ± 0.13	1.00 ± 0.02	1.00 ± 0.09	1.00 ± 0.08
	5 µM Cd	0.86 ± 0.04	1.18 ± 0.07*	1.76 ± 0.07	1.53 ± 0.06
	10 µM Cd	1.34 ± 0.14	1.47 ± 0.15	5.48 ± 0.60	3.88 ± 0.25*

Supplementary Table 6.6. *Metabolic profiling in leaves of wild-type (WT) and aox1a knockout (aox1a) Arabidopsis thaliana plants. (A) Time-dependent changes (0, 24 and 72 h) in metabolite levels compared for unexposed WT and aox1a knockout plants. Data are given as the mean ± S.E. of 6 biological replicates relative to the unexposed WT at 0 h set at 1.00. Different letters represent significant differences within and between both genotypes ($p < 0.05$) (two-way ANOVA). (B) Metabolite levels measured in leaves of WT versus aox1a knockout plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 6 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced abundance changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for higher and lower levels respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$) (two-way ANOVA per time point).*

Supplementary Table 6.6. Part A.

A. Amino acids						
	0 h		**24 h**		**72 h**	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Alanine	1.00 ± 0.38a	1.00 ± 0.37a	1.50 ± 0.36a	1.31 ± 0.29a	1.52 ± 0.49a	1.50 ± 0.32a
β-alanine	1.00 ± 0.12a	0.99 ± 0.07a	0.76 ± 0.04a	0.70 ± 0.04a	0.94 ± 0.11a	0.87 ± 0.05a
Asparagine	1.00 ± 0.23a	1.01 ± 0.26a	0.63 ± 0.12a	0.54 ± 0.07a	0.49 ± 0.09a	0.38 ± 0.08a
Aspartate	1.00 ± 0.05a	1.08 ± 0.11a	0.98 ± 0.06a	0.93 ± 0.08a	1.08 ± 0.14a	0.85 ± 0.03a
GABA	1.00 ± 0.06a	0.91 ± 0.07a	1.45 ± 0.16b	1.20 ± 0.07b	1.17 ± 0.23a,b	1.00 ± 0.11a,b
Glutamate	1.00 ± 0.14a	0.98 ± 0.16a	0.86 ± 0.10a,b	0.78 ± 0.07a,b	0.60 ± 0.11a,b	0.45 ± 0.07b
Glutamine	1.00 ± 0.42a	1.06 ± 0.47a	0.84 ± 0.19a	0.62 ± 0.16a	0.83 ± 0.18a	0.60 ± 0.18a
Glycine	1.00 ± 0.08a,c	1.11 ± 0.11a	0.72 ± 0.05b,c	0.61 ± 0.04b	0.76 ± 0.12b,c	0.70 ± 0.05b,c
Isoleucine	1.00 ± 0.10a	0.99 ± 0.07a	1.05 ± 0.06a	0.91 ± 0.05a	1.03 ± 0.03a	1.04 ± 0.09a
Lysine	1.00 ± 0.03a	1.15 ± 0.07a	1.02 ± 0.06a	0.93 ± 0.04a	1.17 ± 0.12a	0.95 ± 0.06a
Methionine	1.00 ± 0.18a	0.59 ± 0.08a	0.47 ± 0.11a	0.58 ± 0.08a	0.62 ± 0.21a	0.65 ± 0.17a
Ornithine	1.00 ± 0.09a	0.94 ± 0.09a	0.97 ± 0.09a	0.85 ± 0.06a	0.70 ± 0.05a,b	0.53 ± 0.03b
Phenyl-alanine	1.00 ± 0.08a	1.00 ± 0.02a	0.88 ± 0.04a,b	0.77 ± 0.04b,c	0.72 ± 0.05b,c	0.64 ± 0.05c
Proline	1.00 ± 0.10a,b	0.82 ± 0.09a	1.55 ± 0.08b,c	1.66 ± 0.28c	0.69 ± 0.09a	0.67 ± 0.06a
Serine	1.00 ± 0.05a	1.00 ± 0.07a	0.97 ± 0.03a	0.80 ± 0.03a	0.97 ± 0.04a	0.90 ± 0.06a
Threonine	1.00 ± 0.06a	1.01 ± 0.09a,c	1.42 ± 0.03b	1.28 ± 0.05a,b	1.47 ± 0.09b	1.31 ± 0.09b,c
Tryptophan	1.00 ± 0.09a	0.92 ± 0.07a	1.00 ± 0.04a	0.91 ± 0.06a	1.04 ± 0.05a	0.89 ± 0.05a
Valine	1.00 ± 0.09a	0.92 ± 0.05a	0.96 ± 0.04a	0.85 ± 0.06a	0.90 ± 0.07a	0.80 ± 0.06a

Supplementary Table 6.6. Part A. *Continued.*

B. Organic acids

	0 h		24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Citrate	1.00 ± 0.12a	0.90 ± 0.12a	0.82 ± 0.06a	0.74 ± 0.07a	0.79 ± 0.06a	0.69 ± 0.06a
Fumarate	1.00 ± 0.05a	0.83 ± 0.05a,b	0.82 ± 0.07a,b	0.82 ± 0.05a,b	0.79 ± 0.04a,b	0.75 ± 0.06b
Malate	1.00 ± 0.03a	0.98 ± 0.09a	1.19 ± 0.09a,b	1.07 ± 0.11a	1.59 ± 0.17b	1.24 ± 0.13a,b
Pyruvate	1.00 ± 0.15a	0.78 ± 0.14a	1.01 ± 0.26a	1.02 ± 0.19a	0.80 ± 0.20a	1.03 ± 0.30a
Succinate	1.00 ± 0.10a	0.85 ± 0.08a	0.86 ± 0.09a	0.81 ± 0.06a	0.90 ± 0.12a	0.64 ± 0.05a

C. Sugars

	0 h		24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Fructose	1.00 ± 0.22a	0.98 ± 0.15a	5.45 ± 0.38b	5.06 ± 0.47b	4.54 ± 0.31b,c	3.47 ± 0.50c
Glucose	1.00 ± 0.16a	1.12 ± 0.14a	3.39 ± 0.19b	3.16 ± 0.33b	2.73 ± 0.28b,c	2.08 ± 0.29c
Glucoheptose	1.00 ± 0.06a	0.92 ± 0.07a	1.04 ± 0.07a	0.99 ± 0.06a	0.91 ± 0.03a	0.93 ± 0.10a
Maltose	1.00 ± 0.16a	0.89 ± 0.11a	0.12 ± 0.01b	0.11 ± 0.01b	0.16 ± 0.01b	0.12 ± 0.01b
Raffinose	1.00 ± 0.25a	1.12 ± 0.42a	1.24 ± 0.23a	0.93 ± 0.19a	3.91 ± 1.03b	2.66 ± 0.79a,b
Trehalose	1.00 ± 0.08a	0.87 ± 0.06a,b	0.87 ± 0.04a,b	0.84 ± 0.05a,b	0.74 ± 0.08a,b	0.67 ± 0.10b

D. Other metabolites

	0 h		24 h		72 h	
	WT	***aox1a***	**WT**	***aox1a***	**WT**	***aox1a***
Inositol	1.00 ± 0.10a	0.76 ± 0.06a	0.78 ± 0.08a	0.69 ± 0.02a	0.81 ± 0.07a	0.75 ± 0.10a
Putrescine	1.00 ± 0.06a	1.18 ± 0.05a,b	1.34 ± 0.10b	1.20 ± 0.08a,b	1.38 ± 0.26a,b,c	0.79 ± 0.05c
Spermidine	1.00 ± 0.22a	1.02 ± 0.17a	0.91 ± 0.18a	0.91 ± 0.29a	0.94 ± 0.10a	1.02 ± 0.04a
Threonate	1.00 ± 0.19a	1.13 ± 0.26a	1.11 ± 0.24a	0.91 ± 0.13a	1.14 ± 0.21a	0.89 ± 0.16a
Urea	1.00 ± 0.14a	1.19 ± 0.29a	0.70 ± 0.12a,b	0.45 ± 0.05b	0.60 ± 0.07a,b	0.58 ± 0.04a,b

Supplementary Table 6.6. Part B.

A. Amino acids

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Alanine	**Control**	1.00 ± 0.24	1.00 ± 0.22	1.00 ± 0.32	1.00 ± 0.21
	5 µM Cd	1.67 ± 0.32	2.28 ± 0.44	1.33 ± 0.22	2.53 ± 0.69
	10 µM Cd	1.87 ± 0.36	3.01 ± 0.50	0.94 ± 0.25	2.80 ± 0.85
β-alanine	**Control**	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.12	1.00 ± 0.06
	5 µM Cd	5.25 ± 0.29	6.15 ± 0.46	4.27 ± 0.18	4.60 ± 0.30
	10 µM Cd	6.95 ± 0.61	8.81 ± 0.58	3.97 ± 0.28	6.94 ± 0.51*
Asparagine	**Control**	1.00 ± 0.20	1.00 ± 0.14	1.00 ± 0.19	1.00 ± 0.20
	5 µM Cd	1.67 ± 0.24	2.10 ± 0.44	15.13 ± 3.37	14.98 ± 3.17
	10 µM Cd	2.68 ± 0.56	2.73 ± 0.57	13.86 ± 3.11	22.49 ± 5.10
Aspartate	**Control**	1.00 ± 0.06	1.00 ± 0.08	1.00 ± 0.13	1.00 ± 0.04
	5 µM Cd	1.05 ± 0.04	1.30 ± 0.10	1.76 ± 0.25	2.26 ± 0.18
	10 µM Cd	1.47 ± 0.19	1.17 ± 0.09	2.49 ± 0.10	2.88 ± 0.23
GABA	**Control**	1.00 ± 0.11	1.00 ± 0.06	1.00 ± 0.20	1.00 ± 0.12
	5 µM Cd	1.83 ± 0.15	1.88 ± 0.16	2.09 ± 0.24	4.14 ± 0.97
	10 µM Cd	1.94 ± 0.06	3.22 ± 0.43*	3.17 ± 0.66	7.08 ± 0.29
Glutamate	**Control**	1.00 ± 0.12	1.00 ± 0.09	1.00 ± 0.19	1.00 ± 0.15
	5 µM Cd	1.22 ± 0.13	1.43 ± 0.20	2.44 ± 0.31	2.78 ± 0.39
	10 µM Cd	1.45 ± 0.15	1.38 ± 0.19	3.69 ± 0.34	4.48 ± 0.73
Glutamine	**Control**	1.00 ± 0.23	1.00 ± 0.26	1.00 ± 0.21	1.00 ± 0.31
	5 µM Cd	1.25 ± 0.25	2.71 ± 0.58	3.87 ± 0.95	6.99 ± 1.18
	10 µM Cd	2.33 ± 0.43	2.85 ± 0.54	3.14 ± 0.66	12.70 ± 2.77*
Glycine	**Control**	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.16	1.00 ± 0.07
	5 µM Cd	4.23 ± 0.34	4.46 ± 0.51	4.49 ± 0.27	4.95 ± 0.32
	10 µM Cd	4.16 ± 0.53	7.63 ± 0.87*	3.99 ± 0.42	11.12 ± 0.63*

Supplementary Table 6.6. Part B. *Continued.*

A. Amino acids (continued)					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Isoleucine	**Control**	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.03	1.00 ± 0.09
	5 µM Cd	5.74 ± 0.36	6.77 ± 0.92	6.29 ± 1.08	6.62 ± 0.72
	10 µM Cd	7.19 ± 0.70	13.38 ± 1.34*	10.43 ± 2.44	15.37 ± 0.89
Lysine	**Control**	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.10	1.00 ± 0.06
	5 µM Cd	3.04 ± 0.22	3.25 ± 0.37	4.96 ± 0.46	6.96 ± 0.53
	10 µM Cd	4.05 ± 0.55	7.24 ± 0.61*	4.66 ± 0.97	9.43 ± 0.76*
Methionine	**Control**	1.00 ± 0.23	1.00 ± 0.14	1.00 ± 0.34	1.00 ± 0.27
	5 µM Cd	1.88 ± 0.18	2.38 ± 0.58	4.37 ± 0.56	4.81 ± 0.43
	10 µM Cd	2.25 ± 0.19	2.69 ± 0.31	4.12 ± 0.20	6.63 ± 0.80
Ornithine	**Control**	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.06
	5 µM Cd	1.46 ± 0.09	1.66 ± 0.19	9.27 ± 1.18	10.34 ± 1.80
	10 µM Cd	2.07 ± 0.09	2.57 ± 0.11*	7.57 ± 0.64	17.57 ± 2.48*
Phenylalanine	**Control**	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.08
	5 µM Cd	3.31 ± 0.20	3.98 ± 0.38	4.06 ± 0.67	5.06 ± 0.54
	10 µM Cd	3.98 ± 0.32	6.37 ± 0.45*	5.79 ± 1.21	10.86 ± 0.99*
Proline	**Control**	1.00 ± 0.05	1.00 ± 0.17	1.00 ± 0.13	1.00 ± 0.09
	5 µM Cd	1.01 ± 0.15	1.06 ± 0.11	8.27 ± 0.54	10.28 ± 1.23
	10 µM Cd	0.87 ± 0.05	1.02 ± 0.07	9.36 ± 1.06	13.86 ± 1.52
Serine	**Control**	1.00 ± 0.03	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.07
	5 µM Cd	3.26 ± 0.16	4.48 ± 0.34*	4.57 ± 0.14	5.07 ± 0.36
	10 µM Cd	3.27 ± 0.09	4.89 ± 0.34*	4.60 ± 0.23	7.26 ± 0.55*
Threonine	**Control**	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.07
	5 µM Cd	1.95 ± 0.09	2.47 ± 0.18	2.57 ± 0.09	3.03 ± 0.16
	10 µM Cd	1.99 ± 0.12	2.78 ± 0.17*	1.93 ± 0.19	3.03 ± 0.18*

Supplementary Table 6.6. Part B. *Continued.*

A. Amino acids (continued)					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Tryptophan	**Control**	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.05	1.00 ± 0.06
	5 µM Cd	1.65 ± 0.08	2.09 ± 0.14	4.07 ± 0.39	7.62 ± 1.06*
	10 µM Cd	2.84 ± 0.09	3.73 ± 0.26*	9.32 ± 1.45	14.97 ± 0.82*
Valine	**Control**	1.00 ± 0.04	1.00 ± 0.07	1.00 ± 0.07	1.00 ± 0.07
	5 µM Cd	3.78 ± 0.17	4.65 ± 0.39	3.36 ± 0.45	4.19 ± 0.33
	10 µM Cd	4.12 ± 0.30	6.66 ± 0.38*	4.61 ± 0.84	8.22 ± 0.56*
B. Organic acids					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Citrate	**Control**	1.00 ± 0.07	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.09
	5 µM Cd	1.42 ± 0.08	1.45 ± 0.09	4.58 ± 0.56	6.31 ± 0.68
	10 µM Cd	2.83 ± 0.48	2.02 ± 0.27	9.13 ± 0.68	11.42 ± 1.01
Fumarate	**Control**	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.05	1.00 ± 0.08
	5 µM Cd	1.66 ± 0.05	1.77 ± 0.17	2.52 ± 0.20	2.21 ± 0.15
	10 µM Cd	2.49 ± 0.24	2.16 ± 0.20	4.01 ± 0.28	3.85 ± 0.22
Malate	**Control**	1.00 ± 0.08	1.00 ± 0.10	1.00 ± 0.11	1.00 ± 0.11
	5 µM Cd	1.83 ± 0.13	2.16 ± 0.18	5.46 ± 0.38	6.60 ± 0.43
	10 µM Cd	3.23 ± 0.38	2.60 ± 0.31	13.78 ± 1.75	18.27 ± 1.97
Pyruvate	**Control**	1.00 ± 0.26	1.00 ± 0.18	1.00 ± 0.25	1.00 ± 0.29
	5 µM Cd	0.62 ± 0.16	0.80 ± 0.24	1.41 ± 0.17	1.35 ± 0.12
	10 µM Cd	0.56 ± 0.15	0.83 ± 0.31	1.22 ± 0.22	1.14 ± 0.18
Succinate	**Control**	1.00 ± 0.10	1.00 ± 0.07	1.00 ± 0.13	1.00 ± 0.08
	5 µM Cd	2.50 ± 0.19	2.55 ± 0.14	2.11 ± 0.09	3.59 ± 0.30*
	10 µM Cd	3.26 ± 0.24	3.94 ± 0.35	3.51 ± 0.24	5.94 ± 0.33*

Supplementary Table 6.6. Part B. *Continued.*

C. Sugars					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Fructose	**Control**	1.00 ± 0.07	1.00 ± 0.09	1.00 ± 0.07	1.00 ± 0.14
	5 µM Cd	2.25 ± 0.20	2.55 ± 0.37	2.36 ± 0.33	2.93 ± 0.43
	10 µM Cd	2.83 ± 0.30	3.62 ± 0.32	7.96 ± 0.99	12.66 ± 0.58
Glucose	**Control**	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.10	1.00 ± 0.14
	5 µM Cd	2.23 ± 0.26	2.40 ± 0.44	3.28 ± 0.67	4.49 ± 0.76
	10 µM Cd	3.00 ± 0.35	4.17 ± 0.50	17.23 ± 4.08	28.14 ± 3.52
Glucoheptose	**Control**	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.11
	5 µM Cd	1.19 ± 0.05	1.53 ± 0.13	1.57 ± 0.20	1.60 ± 0.16
	10 µM Cd	1.54 ± 0.12	1.79 ± 0.09	3.67 ± 0.60	4.18 ± 0.36
Maltose	**Control**	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.08	1.00 ± 0.09
	5 µM Cd	17.38 ± 2.19	16.56 ± 3.47	1.57 ± 0.16	4.16 ± 0.91*
	10 µM Cd	56.54 ± 6.43	112.77 ± 24.81	8.36 ± 1.86	18.41 ± 5.88
Raffinose	**Control**	1.00 ± 0.19	1.00 ± 0.21	1.00 ± 0.26	1.00 ± 0.30
	5 µM Cd	0.70 ± 0.03	1.49 ± 0.28*	1.29 ± 0.25	1.82 ± 0.25
	10 µM Cd	1.23 ± 0.13	1.43 ± 0.17	3.02 ± 0.45	1.82 ± 0.11
Trehalose	**Control**	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.11	1.00 ± 0.14
	5 µM Cd	0.78 ± 0.07	0.89 ± 0.09	0.44 ± 0.06	0.55 ± 0.05
	10 µM Cd	0.64 ± 0.06	0.69 ± 0.05	0.63 ± 0.06	0.55 ± 0.04

Supplementary Table 6.6. Part B. *Continued.*

D. Other metabolites					
		24 h		**72 h**	
		WT	***aox1a***	**WT**	***aox1a***
Inositol	**Control**	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.09	1.00 ± 0.13
	5 µM Cd	0.80 ± 0.03	1.07 ± 0.08	0.61 ± 0.07	0.70 ± 0.05
	10 µM Cd	0.87 ± 0.04	0.92 ± 0.08	1.06 ± 0.11	0.71 ± 0.01
Putrescine	**Control**	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.19	1.00 ± 0.07
	5 µM Cd	3.67 ± 0.33	4.08 ± 0.18	11.72 ± 0.90	18.03 ± 1.07*
	10 µM Cd	3.21 ± 0.28	5.19 ± 0.30*	4.95 ± 0.28	11.65 ± 1.45*
Spermidine	**Control**	1.00 ± 0.20	1.00 ± 0.32	1.00 ± 0.10	1.00 ± 0.04
	5 µM Cd	0.64 ± 0.17	1.02 ± 0.05	0.31 ± 0.03	0.22 ± 0.02
	10 µM Cd	0.28 ± 0.09	0.78 ± 0.08*	0.20 ± 0.03	0.15 ± 0.01
Threonate	**Control**	1.00 ± 0.21	1.00 ± 0.15	1.00 ± 0.18	1.00 ± 0.18
	5 µM Cd	1.21 ± 0.23	1.52 ± 0.24	1.38 ± 0.12	1.75 ± 0.15
	10 µM Cd	1.63 ± 0.24	1.98 ± 0.34	2.63 ± 0.20	3.34 ± 0.15*
Urea	**Control**	1.00 ± 0.17	1.00 ± 0.10	1.00 ± 0.12	1.00 ± 0.08
	5 µM Cd	1.18 ± 0.14	1.49 ± 0.12	2.06 ± 0.20	2.31 ± 0.41
	10 µM Cd	1.32 ± 0.18	2.17 ± 0.41	1.87 ± 0.16	2.63 ± 0.31

Supplementary Table 6.7. *Relative root transcript levels of genes encoding markers for ROS-induced gene expression, ROS producing and antioxidative enzymes in Arabidopsis thaliana. Transcript levels were measured using quantitative real-time PCR in root samples of 19-days-old wild-type (WT) and aox1a knockout (aox1a) plants exposed to 5 or 10 µM $CdSO_4$ during 24 and 72 h or grown under control conditions. Per time point, data are given as the mean ± S.E. of 4 biological replicates relative to the unexposed genotype set at 1.00. Significant Cd-induced expression changes within each genotype relative to the control are indicated with colour shading: $p < 0.05$; $p < 0.01$ and $p < 0.05$; $p < 0.01$ for induction and inhibition respectively, while differences between both genotypes are indicated with asterisks ($p < 0.05$) (two-way ANOVA per time point). Abbreviations: Supplementary Table 6.1.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding markers for ROS-induced gene expression					
UPOX	**Control**	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.07	1.00 ± 0.10
	5 µM Cd	1.43 ± 0.21	1.80 ± 0.24	2.08 ± 0.36	3.71 ± 0.65
	10 µM Cd	2.71 ± 0.15	3.78 ± 0.55	8.08 ± 1.85	10.77 ± 0.64
Defensin-like	**Control**	1.00 ± 0.12	1.00 ± 0.25	1.00 ± 0.18	1.00 ± 0.13
	5 µM Cd	3.19 ± 0.59	2.79 ± 0.62	28.13 ± 4.40	34.48 ± 16.94
	10 µM Cd	59.31 ± 2.84	59.47 ± 7.87	951.59 ± 7.28	1210.26 ± 192.65
Unknown (AT1G19020)	**Control**	1.00 ± 0.09	1.00 ± 0.15	1.00 ± 0.03	1.00 ± 0.10
	5 µM Cd	3.19 ± 0.42	4.43 ± 0.60	4.14 ± 0.66	5.25 ± 0.88
	10 µM Cd	14.78 ± 0.58	20.93 ± 1.54	19.36 ± 4.26	21.50 ± 2.31
Unknown (AT1G05340)	**Control**	1.00 ± 0.09	1.00 ± 0.16	1.00 ± 0.14	1.00 ± 0.07
	5 µM Cd	1.24 ± 0.12	1.23 ± 0.13	0.82 ± 0.11	1.24 ± 0.26
	10 µM Cd	2.10 ± 0.17	1.38 ± 0.04	2.44 ± 0.26	3.76 ± 0.27
TIR-class	**Control**	1.00 ± 0.18	1.00 ± 0.17	1.00 ± 0.16	1.00 ± 0.01
	5 µM Cd	1.86 ± 0.15	2.59 ± 0.31	3.11 ± 0.86	3.00 ± 0.23
	10 µM Cd	7.73 ± 0.56	15.02 ± 1.29*	23.32 ± 6.41	38.32 ± 5.53

Supplementary Table 6.7. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding ROS producing enzymes					
	Control	1.00 ± 0.02	1.00 ± 0.10	1.00 ± 0.05	1.00 ± 0.11
LOX1	**5 µM Cd**	6.37 ± 1.93	6.23 ± 1.48	5.98 ± 1.54	5.59 ± 1.42
	10 µM Cd	55.59 ± 7.21	60.08 ± 5.13	46.15 ± 5.36	41.69 ± 5.74
	Control	1.00 ± 0.12	1.00 ± 0.09	1.00 ± 0.07	1.00 ± 0.16
LOX4	**5 µM Cd**	10.46 ± 0.34	9.89 ± 0.23	4.26 ± 0.88	5.15 ± 0.88
	10 µM Cd	44.89 ± 1.79	52.69 ± 4.67	75.21 ± 23.55	83.06 ± 16.50
	Control	1.00 ± 0.05	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.08
LOX5	**5 µM Cd**	0.70 ± 0.05	0.67 ± 0.04	0.56 ± 0.03	0.52 ± 0.03
	10 µM Cd	0.87 ± 0.04	0.88 ± 0.02	0.93 ± 0.24	0.98 ± 0.20
	Control	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.04	1.00 ± 0.03
LOX6	**5 µM Cd**	1.19 ± 0.07	1.18 ± 0.08	1.10 ± 0.05	1.08 ± 0.06
	10 µM Cd	1.95 ± 0.06	2.45 ± 0.08*	2.36 ± 0.32	2.72 ± 0.38
	Control	1.00 ± 0.00	1.00 ± 0.08	1.00 ± 0.13	1.00 ± 0.01
RBOHC	**5 µM Cd**	0.73 ± 0.05	0.75 ± 0.07	0.54 ± 0.02	0.76 ± 0.07
	10 µM Cd	0.39 ± 0.00	0.51 ± 0.05	0.57 ± 0.05	0.56 ± 0.01
	Control	1.00 ± 0.06	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.05
RBOHD	**5 µM Cd**	1.82 ± 0.18	2.18 ± 0.17	1.76 ± 0.13	2.39 ± 0.25
	10 µM Cd	3.25 ± 0.18	4.44 ± 0.12	3.46 ± 0.07	5.47 ± 0.60*
	Control	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.01	1.00 ± 0.05
RBOHF	**5 µM Cd**	1.74 ± 0.02	1.75 ± 0.15	1.28 ± 0.10	1.48 ± 0.01
	10 µM Cd	2.06 ± 0.06	2.59 ± 0.01	2.52 ± 0.26	2.67 ± 0.17

Supplementary Table 6.7. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding antioxidative enzymes					
	Control	1.00 ± 0.02	1.00 ± 0.05	1.00 ± 0.08	1.00 ± 0.05
APX1	**5 µM Cd**	0.62 ± 0.03	0.72 ± 0.07	0.72 ± 0.03	0.83 ± 0.03
	10 µM Cd	0.59 ± 0.03	0.73 ± 0.00	1.16 ± 0.11	1.24 ± 0.05
	Control	1.00 ± 0.20	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.04
CAT1	**5 µM Cd**	2.14 ± 0.17	1.80 ± 0.13	1.06 ± 0.07	1.27 ± 0.14
	10 µM Cd	7.59 ± 0.44	6.52 ± 0.65	2.70 ± 0.37	3.53 ± 0.41
	Control	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.14	1.00 ± 0.07
CAT2	**5 µM Cd**	1.04 ± 0.02	1.17 ± 0.05	0.65 ± 0.02	0.72 ± 0.00
	10 µM Cd	0.89 ± 0.00	0.90 ± 0.07	0.43 ± 0.02	0.49 ± 0.03
	Control	1.00 ± 0.08	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.06
CAT3	**5 µM Cd**	1.99 ± 0.38	1.69 ± 0.22	1.58 ± 0.21	1.61 ± 0.18
	10 µM Cd	2.66 ± 0.27	2.74 ± 0.50	3.29 ± 0.17	4.13 ± 0.32
	Control	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.10	1.00 ± 0.07
CSD1	**5 µM Cd**	0.89 ± 0.04	1.09 ± 0.12	0.59 ± 0.02	0.58 ± 0.06
	10 µM Cd	1.60 ± 0.08	1.78 ± 0.13	0.76 ± 0.08	0.73 ± 0.04
	Control	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.06
CSD2	**5 µM Cd**	0.93 ± 0.02	1.06 ± 0.04	0.64 ± 0.03	0.73 ± 0.02
	10 µM Cd	1.23 ± 0.08	1.24 ± 0.05	0.49 ± 0.02	0.49 ± 0.03
	Control	1.00 ± 0.12	1.00 ± 0.03	1.00 ± 0.40	1.00 ± 0.28
FSD1	**5 µM Cd**	18.43 ± 1.10	18.20 ± 1.48	13.83 ± 0.98	20.29 ± 1.49
	10 µM Cd	22.29 ± 2.16	18.54 ± 1.45	20.72 ± 1.58	34.25 ± 2.92
	Control	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.08	1.00 ± 0.01
GR1	**5 µM Cd**	1.16 ± 0.03	1.30 ± 0.07	1.12 ± 0.04	1.28 ± 0.11
	10 µM Cd	2.18 ± 0.12	2.58 ± 0.15	2.65 ± 0.34	2.74 ± 0.15

Supplementary Table 6.7. *Continued.*

		24 h		72 h	
		WT	***aox1a***	**WT**	***aox1a***
Genes encoding mitochondrial antioxidative enzymes					
	Control	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.04
APX	**5 µM Cd**	0.72 ± 0.06	0.76 ± 0.01	0.64 ± 0.06	0.71 ± 0.03
	10 µM Cd	0.92 ± 0.03	1.11 ± 0.00	0.99 ± 0.10	1.08 ± 0.01
	Control	1.00 ± 0.06	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.03
GR2	**5 µM Cd**	1.02 ± 0.02	1.12 ± 0.05	0.79 ± 0.02	1.02 ± 0.06*
	10 µM Cd	1.13 ± 0.05	1.20 ± 0.03	0.89 ± 0.02	1.10 ± 0.06
	Control	1.00 ± 0.05	1.00 ± 0.04	1.00 ± 0.08	1.00 ± 0.09
MDHAR	**5 µM Cd**	0.68 ± 0.06	0.74 ± 0.08	0.53 ± 0.01	0.72 ± 0.05
	10 µM Cd	0.41 ± 0.03	0.49 ± 0.07	0.48 ± 0.02	0.57 ± 0.05
	Control	1.00 ± 0.00	1.00 ± 0.03	1.00 ± 0.06	1.00 ± 0.03
Prx	**5 µM Cd**	1.01 ± 0.04	1.10 ± 0.03	0.84 ± 0.05	1.09 ± 0.02*
	10 µM Cd	1.28 ± 0.04	1.31 ± 0.04	1.02 ± 0.00	1.28 ± 0.05*

CHAPTER 7

General discussion, conclusion and future outlook

7.1. STUDY FRAMEWORK

Worldwide, the metal industry and use of metal-containing fertilisers and pesticides in agriculture contributed significantly to metal pollution, causing diminished crop productivity and economic losses. Contamination with metals is of great concern due to their known toxicity regarding the environment and human health. Cadmium (Cd), a non-essential non-redox-active metal, disturbs physiological processes in plants. At the cellular level, it leads to an oxidative challenge characterised by an imbalance between pro- and antioxidants. Within this framework, mounting evidence indicates that plant mitochondria act in stress perception and response signalling. They represent an important source of reactive oxygen species (ROS) under physiological as well as Cd stress conditions. Literature commonly points towards the mitochondrial alternative oxidase (AOX) as crucial mediator of plant stress responses. It is experimentally proven that AOX can reduce mitochondrial ROS production and alter the cellular energy and metabolic status, which could be important factors influencing the response of plants to Cd.

Details about the critical role mitochondria and more in particular AOX may play during Cd exposure in plants are limited. Therefore, the main objective of the current work was to explore their involvement in the Cd-induced oxidative challenge in *Arabidopsis thaliana*. To this end, sublethal Cd exposure concentrations should be used and thus, the impact of Cd on the ability of *A. thaliana* to complete its life cycle was first determined (Chapter 3). Subsequently, time-course measurements were performed to unravel the relationship between the Cd-induced oxidative challenge, mitochondria and AOX at multiple cellular functional levels (Chapter 4). The specific role of the dominant AOX1a isoform was further explored by comparing Cd stress responses in wild-type (WT) plants and *aox1a* knockout mutants. First, the relationship between AOX1a and the oxidative challenge was studied in leaves of Cd-exposed plants (Chapter 5). Finally, it was investigated if and how AOX1a modulates metabolic responses to Cd in roots and leaves (Chapter 6).

All experiments were conducted using *A. thaliana*, one of the most widely adopted model organisms in plant sciences and especially in molecular research. This is related to the availability of databases mapping genetic and molecular biology information (The Arabidopsis Information Resource). Moreover, its small (125-megabase) genome sequence has been analysed (The Arabidopsis Genome Initiative 2000), thereby expanding the use of molecular tools, transformants and mutants in forward and reverse genetic studies. Within our research group, a hydroponic cultivation setup has been established to study environmental stress conditions in a controlled manner (Smeets *et al*. 2008).

7.2. TISSUE-SPECIFIC RESPONSES TO SUBLETHAL CD EXPOSURE IN WILD-TYPE *ARABIDOPSIS THALIANA* PLANTS

To unravel Cd phytotoxicity mechanisms in *Arabidopsis* roots and leaves, time-course measurements were performed before and 2, 24, 48 and 72 h after the start of Cd exposure. These time points were selected as they include a complete circadian cycle (24 h) and allow studying responses within a short-term (2 h) and more prolonged (48 and 72 h) time frame. Exposure to environmentally realistic (5 or 10 µM) Cd concentrations is toxic to WT *A. thaliana* plants as indicated by altered growth parameters (Figure 4.1) and increased lipid peroxidation (Figure 4.3). However, chronically exposing *Arabidopsis* plants to both 5 and 10 µM Cd still allowed plants to grow, survive and produce siliques containing germinative seeds. The impact of Cd exposure on vegetative growth *i.e.* biomass production was dependent on the dose. Conversely, both concentrations had similar effects on plant regeneration parameters (Chapter 3). Combining our results after short- and long-term exposure to 5 or 10 µM Cd, plants were able to adapt to sublethal Cd exposure, probably before the emergence of the inflorescence meristem. In addition, the sublethal character of both 5 and 10 µM Cd was underpinned by the lack of ultrastructural abnormalities at the mitochondrial level using transmission electron microscopy in our experimental time frame (data not shown).

Taken together, the obtained data clearly indicate a tissue-dependent role for mitochondria and AOX1a during sublethal Cd exposure in *A. thaliana* (Figure 7.1). Comparing the metabolic profiles of Cd-exposed WT *A. thaliana* roots and leaves after 24 and 72 h, a clear difference was revealed at the level of the

tricarboxylic acid (TCA) cycle. While organic acid levels were mainly reduced in roots (Figure 6.3A), the opposite was observed in the leaves (Figure 6.3B). These results suggest a distinctive mitochondrial involvement in Cd stress responses dependent on the tissue. This might be related to the fact that roots represent the first contact site for Cd within the plant and accumulate more Cd as compared to leaves (Figure 4.2). Additionally, Cd-induced root-to-shoot signalling might partly underlie the differential metabolic response observed in both tissues (Figure 7.1).

Plants are equipped with the alternative pathway of electron transport mediated by AOX. As this pathway adjusts the degree of coupling between carbon metabolism and electron transport, the flexibility of the respiratory system is increased (Vanlerberghe *et al*. 2009). The latter might be particularly helpful during stress conditions such as Cd exposure, when plant metabolism must be reconfigured to regain and maintain metabolic homeostasis. It remains challenging however, to determine the molecular components involved in plant acclimation to Cd exposure. Earlier correlations between alternative respiration, AOX and plant tolerance to Cd were based on experiments using Cd concentrations in a highly toxic range (Garmash and Golovko 2009, Duan *et al*. 2010). In our setup, exposing *A. thaliana* plants to environmentally realistic and sublethal Cd concentrations activated AOX at transcript and protein levels within a time frame of 72 h (Chapter 4). In the roots, expression levels of the dominant *AOX1a* isoform were still strongly enhanced after prolonged (72 h) exposure to the highest Cd concentration (Figure 4.6A). On the other hand, transcript levels peaked after 24 h in the leaves. Moreover, expression of the *AOX1d* isoform was highly increased in leaves of Cd-exposed plants (Figure 4.6B), which might be related to Cd-induced senescence (Clifton *et al*. 2006). Increased *AOX1* transcript levels were supported by a transitory rise in AOX protein content in roots (after 2 h) and leaves (after 24 h) (Figure 4.5). In the leaves, the transient nature of AOX induction at both transcript and protein levels supports a signalling function for this enzyme. However, a differential role is plausible in the roots as *AOX1a* transcript levels were still highly increased after 72 h exposure to 10 µM Cd (Figure 7.1). In the hydroponic cultivation system, only the roots are continuously exposed to Cd in a direct way. This might delay the establishment of a new equilibrium in roots as opposed to

leaves, in agreement with the maintained increase in *AOX1a* transcript levels after 72 h in the roots.

It is important to keep in mind that AOX protein levels are not always correlated with the *in vivo* activity of the AOX pathway (Vanlerberghe 2013, Florez-Sarasa *et al*. 2014). Therefore, post-translational regulation of AOX cannot be excluded and deserves further attention. In the inner mitochondrial membrane, the AOX protein exists as a homodimer of which both subunits are reversibly linked by a disulfide bond (McDonald 2008). As the protein is more active in the reduced state, the specific influence of Cd on the mitochondrial and AOX redox state should be further investigated. Once in a reduced form, AOX is sensitive to activation by e.g. pyruvate (Millar *et al*. 1993). However, levels of this organic acid were decreased during Cd exposure in the roots (Figure 6.3A). Therefore, its potential involvement in regulating AOX activity during Cd exposure remains to be determined.

In addition to AOX, plant mitochondria contain alternative NAD(P)H dehydrogenases (NDs) able to oxidise cytosolic NAD(P)H at the external and matrix NAD(P)H at the internal side of the inner membrane. During Cd exposure, a coordinated expression of *AOX1a* and the external *NDB2* isoform was noted in both roots and leaves (Figure 4.4). This is in agreement with data reported by Clifton *et al*. (2005), who observed co-expression of both genes in *Arabidopsis* suspension cells treated with various abiotic stressors over a 24 h time course. Based on our data, we suggest the establishment of a condensed non-phosphorylating electron transport chain (ETC) during sublethal Cd exposure (Figure 7.1). Functioning through cytosolic NDs and AOX, this might represent a path entirely uncoupled from ATP generation (Vanlerberghe 2013). Functionally, both AOX and NDs might cooperate in the acclimation of *Arabidopsis* plants to environmentally realistic Cd exposure by modulating the extent of mitochondrial ROS production. Indeed, it has been demonstrated that leaves of AOX-suppressed tobacco plants show increased $O_2^{\bullet-}$ levels specifically in mitochondria (Cvetkovska and Vanlerberghe 2012). Still, it is important to determine the impact of AOX function at the different subcellular ROS levels and damage versus signalling responses during Cd exposure in specific subcellular compartments.

On the other hand, plant mitochondria contain an array of enzymes and metabolites to counterbalance ROS production (Chapter 1). However, expression levels of genes encoding antioxidative enzymes active in mitochondria such as manganese superoxide dismutase, peroxiredoxin and thioredoxin were only marginally influenced by Cd (Chapter 4). Therefore, we suggest that complementary to AOX, which is put forward as primary defence regulator in mitochondria, other subcellular defence mechanisms against Cd stress centre around Cd complexation and the action of antioxidative enzymes in both roots and leaves. This is confirmed by experiments in a complementary research project (Jozefczak *et al*. 2014) (Figure 7.1).

7.3. Alternative Oxidase1a as a Modulator During Cd Exposure in *Arabidopsis thaliana* Plants

Of the five AOX genes expressed in *A. thaliana*, *AOX1a* is the dominant isoform (Clifton *et al*. 2006). The screening experiment (Chapter 4) supported its potential role during Cd stress as plants were shown to invest in *AOX1a* at transcript and protein levels (Figure 7.1). Therefore, Cd-induced responses of WT plants were compared to those in plants without functional AOX1a protein (*aox1a* knockout mutants). Time-course measurements (24 and 72 h) were performed at multiple cellular functional levels to determine the functional significance of AOX induction during moderate (5 µM) and more severe (10 µM) Cd exposure.

Under control growth conditions, no phenotypic differences were observed between WT and *aox1a* knockout plants (Chapters 5 and 6). In agreement with previous research (Giraud *et al*. 2008), this is in favour of the view that AOX particularly impacts abiotic stress responses. On the other hand, Gandin *et al*. (2014) recently reported the importance of AOX1a to balance energy partitioning between carbon and nitrogen metabolism in *A. thaliana* leaves. However, growth characteristics, chlorophyll content and Rubisco activity were similar for WT and *aox1a* knockout plants. Thus, a lack of AOX1a does not seem to affect normal plant physiology to a great extent (Vanlerberghe *et al*. 2009).

When WT and *aox1a* knockout plants were exposed to sublethal Cd concentrations, Cd uptake (Table 5.2, Chapter 6) and changes in fresh weight (Figure 6.4) were generally similar for both genotypes. By performing

measurements at multiple cellular functional levels, differential responses were noted in roots and leaves of both genotypes exposed to Cd. In roots, a mutual relationship between AOX1a and antioxidative defence mechanisms is suggested at transcript, enzymatic and metabolic levels. In the leaves, AOX1a is proposed to be involved in metabolic as well as redox homeostasis. It is suggested to function in a signalling pathway to (post-transcriptionally) modulate the oxidative challenge during moderate *i.e.* 5 µM Cd exposure (Figure 7.1).

7.4. A Reciprocal Relationship between AOX1a and Antioxidative Defence in Roots of Cd-Exposed *Arabidopsis thaliana* Plants

Compiling our data, AOX1a is suggested to be interrelated with the induction and function of antioxidative defence mechanisms in roots of Cd-exposed *A. thaliana* plants. The oxidative challenge has been assessed in roots of WT and *aox1a* knockout plants during acute (24 h) and more prolonged (72 h) exposure to 5 or 10 µM Cd (Chapter 6). At the transcript level, only marginal differences were observed between both genotypes regarding expression levels of pro- and antioxidative genes (Supplementary Table 6.7). However, roots of *aox1a* knockout mutants displayed higher antioxidative and NAD(P)H-dependent enzyme capacities as compared to those of WT plants, mainly after 72 h exposure to the highest Cd concentration (Table 6.1). Moreover, levels of glutamine (one of the building blocks for glutathione (GSH) biosynthesis) and the antioxidative metabolite ascorbate (AsA) were higher in roots of *aox1a* knockout mutants (Figure 6.6). Combining these data, it is hypothesised that plants without functional AOX1a suffer from increased (oxidative) stress during Cd exposure, at least in roots. The importance of AOX1a in mediating the oxidative challenge is further underlined by its higher transcriptional induction in roots of GSH-deficient Cd-exposed *cad2-1*, *pad2-1* and *rax1-1* mutants as compared to WT plants (Figure 6.7). On the other hand, no such response was observed in the leaves of these mutants (data not shown). Since GSH is an important Cd chelating agent in *Arabidopsis* roots (Jozefczak *et al*. 2014), mutants deficient in GSH are likely to show increased (oxidative) stress levels during Cd exposure. The increased upregulation of *AOX1a* in these mutants is in favour of the view that AOX modulates the extent of the Cd-induced oxidative challenge in *A. thaliana* roots (Figure 7.1). Recently, expression patterns of

AOX1 and several antioxidant genes were shown to correlate in salt-stressed *Medicago truncatula* roots (Mhadhbi *et al*. 2013). The combination of antioxidative defence and the AOX pathway is suggested to control the oxidative balance, thereby contributing to salt stress tolerance (Mhadhbi *et al*. 2013).
Regarding the mechanisms underlying *AOX1a* induction in Cd-exposed roots, interesting results were recently published by Ivanova *et al*. (2014). They have shown a functional antagonistic link between auxin and mitochondrial retrograde signalling, which is involved in regulating *AOX1a* expression in *A. thaliana*. In antimycin A-challenged mutants with impaired auxin homeostasis, *AOX1a* was induced to a greater extent as compared to WT plants. Their findings collectively suggest that auxin signalling negatively regulates mitochondrial retrograde signalling responsible for *AOX1a* induction during abiotic stress (Ivanova *et al*. 2014). Interestingly, it has been shown that Cd exposure results in reduced endogenous auxin levels in poplar (Elobeid *et al*. 2012) and *A. thaliana* (Hu *et al*. 2013). Concurrently, these observations might imply a role for auxin in mediating *AOX1a* induction during Cd exposure as well. In addition, Tognetti *et al*. (2012) discussed the relation between auxin and ROS, which are both strongly affected by Cd exposure. The crosstalk between both is suggested to regulate plant stress responses and adaptation (Tognetti *et al*. 2012).

7.5. Integration of AOX1a, Cd-induced Signalling and Metabolic Homeostasis in *Arabidopsis thaliana* Leaves

In roots, most differences between WT and *aox1a* knockout plants were observed after exposure to 10 µM Cd. On the other hand, AOX1a is suggested to modulate the oxidative challenge during moderate *i.e.* 5 µM Cd exposure in *A. thaliana* leaves (Chapter 5). Based on the assessment of the Cd-induced oxidative challenge at transcript and metabolic (GSH) level, it is suggested that AOX1a functions early (within 72 h) in the response to 5 µM Cd in the leaves. Via AOX, a signal is relayed to other parts of the cell, thereby activating ROS defence pathways. This allows plant cells to reach a new equilibrium between ROS generation and scavenging, a crucial factor in plant acclimation to Cd stress. How this retrograde signal is transduced remains currently unknown, although H_2O_2 might be involved (Figure 7.1). Indeed, its levels were attenuated in leaves of plants lacking functional AOX1a during exposure to 5 µM Cd (Figure

5.2). The retrograde signal initiated by AOX is suggested to impact antioxidative defence mechanisms at the post-transcriptional level. This is in line with the lack of increased GSH levels in leaves of 5 µM Cd-exposed *aox1a* knockout versus WT plants after 72 h (Table 5.3). Moreover, capacities of antioxidative and NAD(P)H-dependent enzymes increased to a lesser extent in leaves of *aox1a* knockout mutants as compared to those of WT plants, mainly after 72 h exposure to 5 µM Cd (data not shown). However, genotype-dependent differences observed during moderate Cd exposure disappeared when comparing responses in leaves of WT and *aox1a* knockout plants exposed to 10 µM Cd (Chapter 5). It is therefore proposed that AOX initiates or maintains a fine-tuning signalling pathway in the leaves during moderate Cd exposure only. During more severe *i.e.* 10 µM Cd stress, this pathway is potentially bypassed by mechanisms related to increased ROS levels. Underlining this, Cd-induced oxidative damage was observed only after 72 h exposure to 10 µM Cd (Figure 4.3). As suggested by Gupta *et al*. (2012), ROS generation by non-mitochondrial sources such as NADPH oxidases could be able to overwhelm any homeostatic action of AOX during 10 µM Cd exposure.

On the other hand, most differences – mainly related to amino acid levels – were detected when comparing the metabolic profiles obtained for leaves of 10 µM Cd-exposed WT versus *aox1a* knockout mutants (Chapter 6). More in particular, levels of lysine, branched chain and aromatic amino acids increased to a higher extent in the absence of functional AOX1a (Figure 6.5). As suggested by Araújo *et al*. (2011), these amino acids might be used to either support the TCA cycle or directly supply electrons to the ubiquinone pool of the mitochondrial ETC. The contribution of this alternative respiration and the potential involvement of AOX in mediating this pathway should further be studied during Cd exposure in *A. thaliana*. A potential link between AOX and branched chain amino acids was demonstrated before in soybean. In response to herbicides inhibiting the first common enzyme in their biosynthetic pathway (acetolactate synthase), the AOX pathway was activated (Gaston *et al*. 2003).

In conclusion, AOX activity is suggested to affect Cd stress responses in *A. thaliana* leaves by modulating redox as well as metabolic homeostasis. Furthermore, our study has shed more light on the genetic control of AOX respiration under Cd stress in the leaves. Using ethylene biosynthesis and

insensitive mutants, the involvement of ethylene – either directly or indirectly through NADPH oxidase-dependent ROS production – in Cd-induced *AOX1* transcriptional activation is unequivocally demonstrated in the leaves (Figure 5.4). Interestingly, γ-aminobutyric acid (GABA) levels were increased to a higher extent in leaves of Cd-exposed *aox1a* knockout versus WT plants after 24 and 72 h exposure to 10 µM Cd (Supplementary Table 6.6). As GABA has been shown to stimulate ethylene biosynthesis (Kathiresan *et al*. 1997), this observation further supports the postulated link between ethylene and AOX during Cd exposure in the leaves. Furthermore, genotype-dependent differences could also be observed for putrescine (Figure 6.5L) and spermidine (Figure 6.5M) levels, both related to Cd-induced production of ethylene (Groppa *et al*. 2003). In conclusion, our data confirm a link between ethylene and AOX, and the involvement of feedback mechanisms during sublethal Cd exposure in *A. thaliana* leaves (Figure 7.1). On the other hand, no such link could be observed in the roots (data not shown). This highlights the fact that perception and response signalling occurs in a tissue-specific manner during Cd exposure in *A. thaliana* (Figure 7.1).

7.6. General Working Model and Conclusion

Compiling the obtained data, a working model depicting the potential tissue-dependent role of mitochondria and more specifically AOX during sublethal Cd exposure in *A. thaliana* is shown in Figure 7.1. In roots, the metabolic profile points towards increased glycolytic and TCA cycle fluxes to supply energy for antioxidative defence mechanisms. In the leaves however, levels of organic acids increased during Cd exposure, supporting a tissue-specific response to Cd (Figure 7.1). Transcript levels of specific mitochondrial antioxidative enzymes either decreased or did not change to a great extent in response to Cd. Therefore, it is more likely that the Cd-induced oxidative challenge at the mitochondrial level is counteracted by the alternative respiratory pathway and the action of cytosolic or other – instead of mitochondrial – antioxidative enzymes, at least at the level of transcription (Figure 7.1).

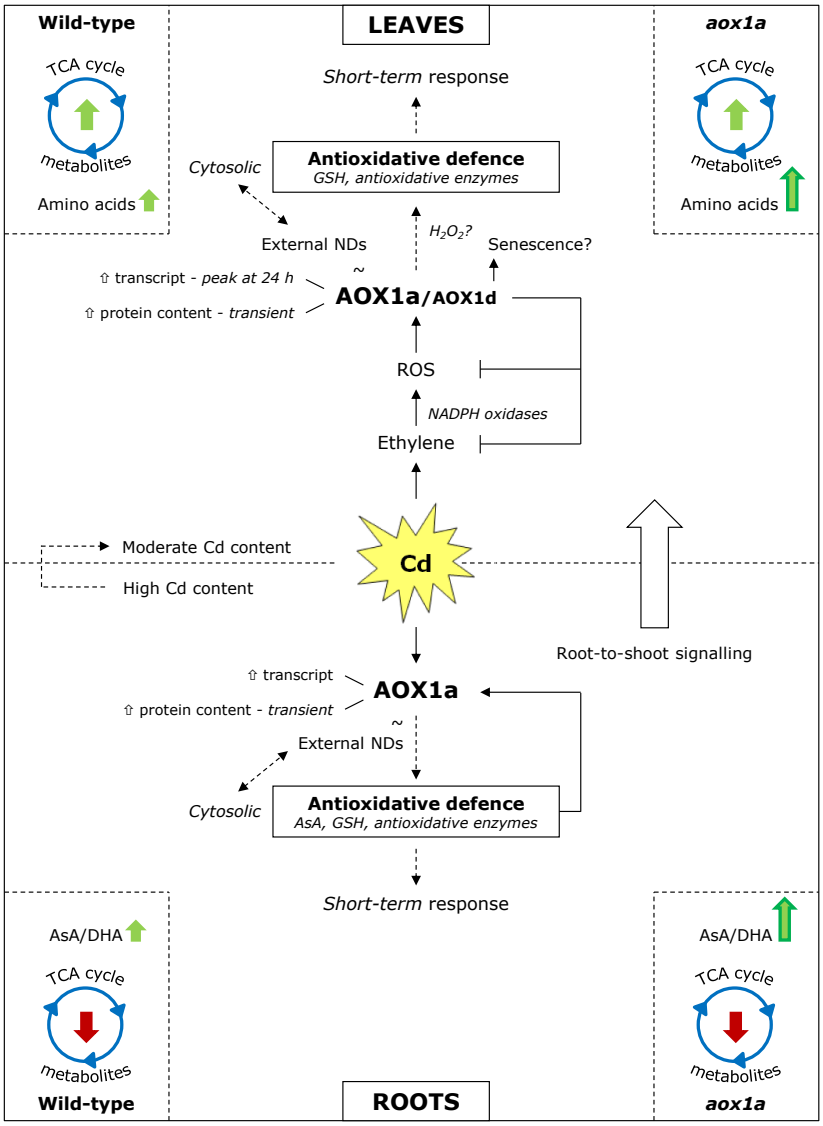


Figure 7.1. *Overview of the proposed working model depicting the potential role of AOX during sublethal Cd exposure in Arabidopsis thaliana roots and leaves. Cadmium exposure differentially affects mitochondrial TCA cycle metabolites in both tissues. In roots, our results suggest a reciprocal relationship between AOX1a and antioxidative defence mechanisms during Cd exposure. In Cd-exposed leaves, ethylene is demonstrated to transcriptionally activate AOX1 genes, potentially via ROS, while AOX1a could exert negative feedback on ethylene and/or ROS. Finally, AOX1a is suggested to function in a fine-tuning signalling pathway in the leaves, thereby activating or maintaining antioxidative defence mechanisms. How the retrograde signal from AOX is transduced remains currently unknown, although H_2O_2 might be involved (see main text for further details). AsA, ascorbate; Cd, cadmium; DHA, dehydroascorbate; GSH, glutathione; H_2O_2, hydrogen peroxide; ND, alternative NAD(P)H dehydrogenase; ROS, reactive oxygen species; TCA, tricarboxylic acid.*

Furthermore, a mutual link between the induction of AOX1a and antioxidative defence mechanisms in roots is suggested within 72 h of Cd exposure. First, capacities of antioxidative defence enzymes and AsA levels increased to a higher extent in the absence of functional AOX1a. On the other hand, a lack of GSH caused enhanced transcriptional activation of *AOX1a* during Cd exposure in roots only. Taken together, AOX1a is suggested to modulate the extent of the oxidative challenge induced by Cd in *A. thaliana* roots at the cellular level (Figure 7.1).

In roots, a lack of AOX1a enhanced the oxidative challenge level induced by 10 µM Cd. In leaves however, AOX1a is suggested to function in a fine-tuning signalling pathway during moderate *i.e.* 5 µM Cd exposure only. This is supported by attenuated H_2O_2, GSH and antioxidative enzyme capacity levels in leaves of 5 µM Cd-exposed *aox1a* knockout mutants as compared to WT plants. This pathway is suggested to be swamped during 10 µM Cd exposure, potentially related to increasing ROS production and oxidative damage. Further, a link is observed between AOX function and amino acid levels, which could point towards stress-induced respiration based on protein degradation. Finally, ethylene (potentially via ROS) is required to fully induce *AOX1* genes at the transcript level in Cd-exposed leaves, with AOX1a potentially exerting negative feedback on ethylene biosynthesis and/or signalling (Figure 7.1).

Although a lack of AOX1a altered Cd-induced responses within a time frame of 72 h after the exposure started, chronically exposing WT and *aox1a* knockout plants to 5 or 10 µM Cd did not reveal any significant differences between both genotypes. Exposure to Cd led to similar decreases in rosette diameter, inflorescence height and number of siliques independent of the genotype (data not shown). However, the shift from vegetative to regenerative growth was fastened in 5 µM Cd-exposed *aox1a* knockout mutants as compared to WT plants, albeit not significantly (Figure 7.2). These results are in line with the suggested Cd-induced accelerated senescence in plants, which possibly occurs earlier in the absence of AOX1a. As discussed before, induction of the *AOX1d* isoform might be related to Cd-induced senescence (Clifton *et al*. 2006). Interestingly, expression levels of *AOX1d* augmented to a greater extent after prolonged (72 h) Cd exposure in the leaves of *aox1a* knockout as compared to WT plants (Figure 5.1B). In turn, this could be linked to the accelerated shift

between vegetative and regenerative growth without functional AOX1a (Figure 7.2), pointing towards a relationship between acute (up to 72 h) and long-term Cd-induced effects in *A. thaliana*. Although future research is certainly required, our results so far are more suggestive of a role for AOX1a during the short-term response to sublethal Cd exposure (Figure 7.1).

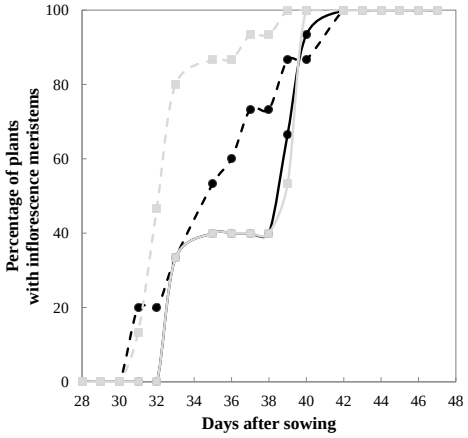


Figure 7.2. *Exposure to Cd has an influence on inflorescence emergence in Arabidopsis thaliana wild-type or aox1a knockout plants continuously exposed to 5 µM $CdSO_4$ after 19 days of growth (WT, dashed black line; aox1a, dashed grey line) or grown under control conditions (WT, black line; aox1a, grey line) The percentage of plants (15 biological replicates per group) with inflorescence meristems are shown over time.*

7.7. FUTURE OUTLOOK

Although our results support a role for AOX during sublethal Cd exposure at transcript and protein levels, the engagement of the alternative respiratory pathway should also be uncovered at the activity level itself. To this end, the actual flux of electrons to AOX or respiratory complex IV can be compared using the oxygen isotope discrimination technique (Guy *et al*. 1989, Ribas-Carbo *et al*. 1995, Robinson *et al*. 1995). To further unravel the potential relationship between AOX, ethylene and GSH, the extent of alternative respiration should be determined in WT versus ethylene biosynthesis and signalling mutants as well as GSH-deficient plants exposed to Cd.

Furthermore, our results are in favour of a relationship between mitochondrial retrograde signals e.g. mediated by AOX via H_2O_2, and GSH. Concentrations and redox state of GSH are suggested to be involved in transducing oxidative signals originating from ROS to activate antioxidative defence mechanisms during Cd exposure. Studying this link might be especially important in Cd-exposed roots, as GSH is directly consumed for chelation purposes, thereby compromising its antioxidant role (Jozefczak *et al*. 2014). Future research should be devoted to unravelling the relationship between mitochondria, AOX and GSH within the

subcellular range, e.g. using specific redox-sensitive probes targeted to *Arabidopsis* mitochondria (Schwarzländer *et al*. 2009). Furthermore, the exact sequence of events can only be determined by narrowing the experimental time frame as well as lowering the Cd concentrations applied in future experiments.

In addition to modulating ROS levels, Cvetkovska and Vanlerberghe (2012) showed that AOX is also able to decrease NO production in transgenic tobacco leaves. As growing evidence indicates that Cd exposure modulates NO generation in plants (Chmielowska-Bąk *et al*. 2014), the potential involvement of AOX in mediating NO homeostasis might be included in future studies.

Recently, more research is devoted to unravelling the modulators of plant mitochondrial retrograde signalling pathways using *AOX1a* expression as a marker (Chapter 1). The involvement of transcription factors such as ANAC013, ANAC017 and WRKY40 during Cd stress responses is plausible but yet remains to be revealed.

In the present study, tissue-specific responses in roots versus leaves to sublethal Cd exposure were evident. However, it is challenging to focus on different cell types within these tissues and thereby continuously increase the experimental resolution to study Cd-induced responses. The study of Van Belleghem *et al*. (2007) providing subcellular information on Cd localisation in *A. thaliana* roots and leaves serves as starting point for future research.

As the maintenance of the mitochondrial ETC is suggested to be a central event in stress tolerance, AOX levels and/or activity might be regarded as potential bio-indicators in search of plants with an increased Cd tolerance. However, to imply this in the development or adjustment of strategies to cope with metal-polluted soils, it is important to also take multipollution into account. In addition, the involvement of epigenetic mechanisms in Cd stress responses and potential acclimation over different plant generations represents a challenging topic for future research.

REFERENCES

Araújo, W.L., Tohge, T., Ishizaki, K., Leaver, C.J. and Fernie, A.R. (2011) Protein degradation – an alternative respiratory substrate for stressed plants. *Trends Plant Sci*, **16**, 489–498.

Chmielowska-Bąk, J., Gzyl, J., Rucińska-Sobkowiak, R., Arasimowicz-Jelonek, M. and Deckert, J. (2014) The new insights into cadmium sensing. *Front Plant Sci*, **5**, 245.

Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A. and Whelan, J. (2005) Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol Biol*, **58**, 193–212.

Clifton, R., Millar, A.H. and Whelan, J. (2006) Alternative oxidases in Arabidopsis: A comparative analysis of differential expression in the gene family provides new insights into function of non-phosphorylating bypasses. *Biochim Biophys Acta*, **1757**, 730–741.

Cvetkovska, M. and Vanlerberghe, G.C. (2012) Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol*, **195**, 32–39.

Duan, Y.P., Yuan, S., Tu, S.H., Feng, W.Q., Xu, F., Zhang, Z.W., Chen, Y.E., Wang, X., Shang, J. and Lin, H.H. (2010) Effects of cadmium stress on alternative oxidase and photosystem II in three wheat cultivars. *Z Naturforsch C*, **65**, 87–94.

Elobeid, M., Göbel, C., Feussner, I. and Polle, A. (2012) Cadmium interferes with auxin physiology and lignification in poplar. *J Exp Bot*, **63**, 1413–1421.

Florez-Sarasa, I., Lambers, H., Wang, X., Finnegan, P.M. and Ribas-Carbo, M. (2014) The alternative respiratory pathway mediates carboxylate synthesis in white lupin cluster roots under phosphorus deprivation. *Plant Cell Environ*, **37**, 922–928.

Gandin, A., Denysyuk, M. and Cousins, A.B. (2014) Disruption of the mitochondrial alternative oxidase (AOX) and uncoupling protein (UCP) alters rates of foliar nitrate and carbon assimilation in *Arabidopsis thaliana*. *J Exp Bot*, **65**, 3133–3142.

Garmash, E.V. and Golovko, T.K. (2009) Effect of cadmium on growth and respiration of barley plants grown under two temperature regimes. *Russ J Plant Physiol*, **56**, 343–347.

Gaston, S., Ribas-Carbo, M., Busquets, S., Berry, J.A., Zabalza, A. and Royuela, M. (2003) Changes in mitochondrial electron partitioning in response to herbicides inhibiting branched-chain amino acid biosynthesis in soybean. *Plant Physiol*, **133**, 1351–1359.

Giraud, E., Ho, L.H.M., Clifton, R., Carroll, A., Estavillo, G., Tan, Y.F., Howell, K.A., Ivanova, A., Pogson, B.J., Millar, A.H. and Whelan, J. (2008) The absence of ALTERNATIVE OXIDASE1a in Arabidopsis results in acute sensitivity to combined light and drought stress. *Plant Physiol*, **147**, 595–610.

Groppa, M.D., Benavides, M.P. and Tomaro, M.L. (2003) Polyamine metabolism in sunflower and wheat leaf discs under cadmium or copper stress. *Plant Sci*, **164**, 293–299.

Gupta, K.J., Igamberdiev, A.U. and Mur, L.A.J. (2012) NO and ROS homeostasis in mitochondria: a central role for alternative oxidase. *New Phytol*, **195**, 1–3.

Guy, R.D., Berry, J.A., Fogel, M.L. and Hoering, T.C. (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. *Planta*, **177**, 483–491.

Hu, Y.F., Zhou, G., Na, X.F., Yang, L., Nan, W.B., Liu, X., Zhang, Y.Q., Li, J.L. and Bi, Y.R. (2013) Cadmium interferes with maintenance of auxin homeostasis in *Arabidopsis* seedlings. *J Plant Physiol*, **170**, 965–975.

Ivanova, A., Law, S.R., Narsai, R., Duncan, O., Lee, J.H., Zhang, B., Van Aken, O., Radomiljac, J.D., van der Merwe, M., Yi, K. and Whelan, J. (2014) A functional antagonistic relationship between auxin and mitochondrial retrograde signaling regulates ALTERNATIVE OXIDASE1a expression in Arabidopsis thaliana. *Plant Physiol*, **165**, 1233–1254.

Jozefczak, M., Keunen, E., Schat, H., Bliek, M., Hernández, L.E., Carleer, R., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A. (2014) Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. *Plant Physiol Biochem*, **83**, 1–9.

Kathiresan, A., Tung, P., Chinnappa, C.C. and Reid, D.M. (1997) γ-aminobutyric acid stimulates ethylene biosynthesis in sunflower. *Plant Physiol*, **115**, 129–135.

McDonald, A.E. (2008) Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed 'cyanide-resistant' terminal oxidase. *Funct Plant Biol*, **35**, 535–552.

Mhadhbi, H., Fotopoulos, V., Mylona, P.V., Jebara, M., Aouani, M.E. and Polidoros, A.N. (2013) Alternative oxidase 1 (*Aox1*) gene expression in roots of *Medicago truncatula* is a genotype-specific component of salt stress tolerance. *J Plant Physiol*, **170**, 111–114.

Millar, A.H., Wiskich, J.T., Whelan, J. and Day, D.A. (1993) Organic acid activation of the alternative oxidase of plant mitochondria. *FEBS Lett*, **329**, 259–262.

Ribas-Carbo, M., Berry, J.A., Yakir, D., Giles, L., Robinson, S.A., Lennon, A.M. and Siedow, J.N. (1995) Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. *Plant Physiol*, **109**, 829–837.

Robinson, S.A., Ribas-Carbo, M., Yakir, D., Giles, L., Reuveni, Y. and Berry, J.A. (1995) Beyond SHAM and cyanide: Opportunities for studying the alternative oxidase in plant respiration using oxygen isotope discrimination. *Aust J Plant Physiol*, **22**, 487–496.

Schwarzländer, M., Fricker, M.D. and Sweetlove, L.J. (2009) Monitoring the *in vivo* redox state of plant mitochondria: Effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. *Biochim Biophys Acta*, **1787**, 468–475.

Smeets, K., Ruytinx, J., Van Belleghem, F., Semane, B., Lin, D., Vangronsveld, J. and Cuypers, A. (2008) Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. *Plant Physiol Biochem*, **46**, 212–218.

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.

Tognetti, V.B., Mühlenbock, P. and Van Breusegem, F. (2012) Stress homeostasis – the redox and auxin perspective. *Plant Cell Environ*, **35**, 321–333.

Van Belleghem, F., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, J., d'Haen, J. and Valcke R. (2007) Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytol*, **173**, 495–508.

Vanlerberghe, G.C., Cvetkovska, M. and Wang, J. (2009) Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? *Physiol Plant*, **137**, 392–406.

Vanlerberghe, G.C. (2013) Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int J Mol Sci*, **14**, 6805–6847.

SCIENTIFIC OUTPUT

INTERNATIONAL JOURNALS

- **Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A.R., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J. and Smeets, K.** (2010) Cadmium stress: an oxidative challenge. *Biometals*, **23**, 927–940.
- **Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J. and Vangronsveld, J.** (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J Plant Physiol*, **168**, 309–316.
- **Keunen, E., Truyens, S., Bruckers, L., Remans, T., Vangronsveld, J. and Cuypers, A.** (2011) Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged? *Plant Physiol Biochem*, **49**, 1084–1091.
- **Keunen, E., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A.** (2011) Metal-induced oxidative stress and plant mitochondria. *Int J Mol Sci*, **12**, 6894–6918.
- **Seth, C.S., Remans, T., Keunen, E., Jozefczak, M., Gielen, H., Opdenakker, K., Weyens, N., Vangronsveld, J. and Cuypers, A.** (2012) Phytoextraction of toxic metals: a central role for glutathione. *Plant Cell Environ*, **35**, 334–346.
- **Remans, T., Thijs, S., Truyens, S., Weyens, N., Schellingen, K., Keunen, E., Gielen, H., Cuypers, A. and Vangronsveld, J.** (2012) Understanding the development of roots exposed to contaminants and the potential of plant-associated bacteria for optimization of growth. *Ann Bot*, **110**, 239–252.
- **Opdenakker, K., Remans, T., Keunen, E., Vangronsveld, J. and Cuypers, A.** (2012) Exposure of *Arabidopsis thaliana* to Cd or Cu excess leads to oxidative stress mediated alterations in MAPKinase transcript levels. *Environ Exp Bot*, **83**, 53–61.
- **Keunen, E., Remans, T., Opdenakker, K., Jozefczak, M., Gielen, H., Guisez, Y., Vangronsveld, J. and Cuypers, A.** (2013) A mutant of the *Arabidopsis thaliana* LIPOXYGENASE1 gene shows altered signalling and oxidative stress related responses after cadmium exposure. *Plant Physiol Biochem*, **63**, 272–280.
- **Keunen, E., Peshev, D., Vangronsveld, J., Van den Ende, W. and Cuypers, A.** (2013) Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. *Plant Cell Environ*, **36**, 1242–1255.

- **Keunen, E., Jozefczak, M., Remans, T., Vangronsveld, J. and Cuypers, A.** (2013) Alternative respiration as a primary defence during cadmium-induced mitochondrial oxidative challenge in *Arabidopsis thaliana*. *Environ Exp Bot*, **91**, 63–73.
- **Nair, A.R., Smeets, K., Keunen, E., Lee, W.K., Thévenod, F., Van Kerkhove, E. and Cuypers, A.** (2014) Renal cells exposed to cadmium *in vitro* and *in vivo*: normalizing gene expression data. *J Appl Toxicol*, doi: 10.1002/jat.3047.
- **Jozefczak, M., Keunen, E., Schat, H., Bliek, M., Hernández, L.E., Carleer, R., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A.** (2014) Differential response of *Arabidopsis* leaves and roots to cadmium: Glutathione-related chelating capacity *vs* antioxidant capacity. *Plant Physiol Biochem*, **83**, 1–9.
- **Remans, T., Keunen, E., Bex, G.J., Smeets, K., Vangronsveld, J. and Cuypers, A.** (2014) Reliable gene expression analysis by reverse transcription-quantitative PCR: Reporting and minimizing the uncertainty in data accuracy. *Plant Cell*, doi: 10.1105/tpc.114.130641.
- **Jozefczak, M., Keunen, E., Schat, H., Bliek, M., Remans, T., Guisez, Y., Vangronsveld, J. and Cuypers, A.** (2014) Deficiency in ascorbate is compensated by glutathione in cadmium-exposed *Arabidopsis* mutants but glutathione deficiency demands for multiple alternatives. Submitted to *New Phytol*.
- **Keunen, E., Schellingen, K., Van Der Straeten, D., Remans, T., Vangronsveld, J. and Cuypers, A.** (2014) ALTERNATIVE OXIDASE1a modulates the oxidative challenge during moderate Cd exposure in *Arabidopsis thaliana* leaves. Submitted to *J Exp Bot*.
- **Keunen, E., Florez-Sarasa, I., Obata, T., Jozefczak, M., Remans, T., Vangronsveld, J., Fernie, A.R. and Cuypers, A.** (2014) Metabolic responses of *Arabidopsis thaliana* roots and leaves to sublethal Cd exposure are differentially influenced by ALTERNATIVE OXIDASE1a. In preparation.

BOOK CHAPTER

- **Cuypers, A., Keunen, E., Bohler, S., Jozefczak, M., Opdenakker, K., Gielen, H., Vercampt, H., Bielen, A., Schellingen, K., Vangronsveld, J. and Remans, T.** (2012) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling, in: Gupta, D.K., Sandalio, L.M. (Eds.), Metal toxicity in plants: Perception, signaling and remediation. Springer-Verlag GmbH, Berlin, Heidelberg, pp. 65–90.

ABSTRACTS

- **Opdenakker, K., Keunen, E., Remans, T., Smeets, K., Vangronsveld, J. and Cuypers, A.** (2008) Molecular mechanisms of heavy metal pollution in *Arabidopsis thaliana*. Advances in qPCR Conference. Stockholm, Sweden. September 16th to 18th 2008. Abstract of poster presentation.
- **Opdenakker, K., Keunen, E., Remans, T., Smeets, K., Vangronsveld, J. and Cuypers, A.** (2008) Molecular mechanisms of heavy metal pollution in *Arabidopsis thaliana*. 1st Benelux qPCR Symposium. Ghent, Belgium. October 6th 2008. Abstract of poster presentation.
- **Remans, T., Gielen, H., Opdenakker, K., Jozefczak, M., Keunen, E., Vangronsveld J. and Cuypers, A.** (2009) Elucidating the molecular triggers of root developmental responses to heavy metal stress. Plant abiotic stress – from signaling to development. Tartu, Estonia. May 14th to 17th 2009. Abstract of oral and poster presentation.
- **Cuypers, A., Remans, T., Opdenakker, K., Jozefczak, M., Keunen, E., Gielen, H. and Vangronsveld, J.** (2009) The cellular redox state as an important modulator in metal stress: metal-specific responses in a multipollution context. COST Action 859. The final international conference on phytotechnologies to promote sustainable land use and food safety. Monte Verità, Ascona, Switzerland. October 11th to 16th 2009. Abstract of oral presentation.
- **Cuypers, A., Smeets, K., Opdenakker, K., Remans, T., Gielen, H., Jozefczak, M., Keunen, E. and Vangronsveld, J.** (2010) Gene expression profiles of antioxidative enzymes and related miRNAs as a tool to identify metal-specific effects in *Arabidopsis thaliana*. Keystone Symposium. RNA silencing mechanisms in Plants. Santa Fe, New Mexico, USA. February 21st to 26th 2010. Abstract of poster presentation.
- **Keunen, E., Jozefczak, M., Gielen, H., Opdenakker, K., Remans, T., Vangronsveld, J. and Cuypers, A.** (2010) Mitochondrial implications during heavy metal toxicity in *Arabidopsis thaliana*. 24th New Phytologist Symposium: Plant respiration and climate change: scaling from mitochondria to the globe. Oxford, UK. April 11th to 14th 2010. Abstract of poster presentation.
- **Cuypers, A., Remans, T., Keunen, E., Weyens, N. and Vangronsveld, J.** (2011) Metal stress: how to overcome the oxidative challenge? Keystone symposium on molecular and cellular biology. Plant abiotic stress tolerance mechanisms, water and global agriculture. Keystone, Colorado, USA. January 17th to 22nd 2011. Abstract of poster presentation.

- **Bielen, A., Schellingen, K., Keunen, E., Jozefczak, M., Opdenakker, K., Vercampt, H., Gielen, H., Remans, T., Vangronsveld, J. and Cuypers, A.** (2011) The role of ascorbate peroxidase 1 during metal-induced oxidative stress in *Arabidopsis thaliana* plants. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest, Hungary. July 5th to 8th 2011. Abstract of poster presentation.
- **Jozefczak, M., Schat, H., Remans, T., Keunen, E., Schellingen, K., Gielen, H., Opdenakker, K., Bielen, A., Vercampt, H., Vangronsveld, J. and Cuypers, A.** (2011) Cadmium-specific responses in *Arabidopsis thaliana*. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest, Hungary. July 5th to 8th 2011. Abstract of poster presentation.
- **Keunen, E., Remans, T., Jozefczak, M., Gielen, H., Opdenakker, K., Vangronsveld, J. and Cuypers, A.** (2011) Mitochondria and cadmium-induced oxidative stress in *Arabidopsis thaliana*. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest, Hungary. July 5th to 8th 2011. Abstract of poster presentation.
- **Opdenakker, K., Remans, T., Keunen, E., Jozefczak, M., Gielen, H., Bielen, A., Schellingen, K., Vercampt, H., Vangronsveld, J. and Cuypers, A.** (2011) MPK3 and MPK6 play a role in metal-induced ROS signaling in *Arabidopsis thaliana*. 10th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Budapest, Hungary. July 5th to 8th 2011. Abstract of poster presentation.
- **Keunen, E., Remans, T., Jozefczak, M., Gielen, H., Opdenakker, K., Vangronsveld, J. and Cuypers, A.** (2011) Mitochondria and cadmium-induced oxidative stress in *Arabidopsis thaliana*. FEBS Workshop: Plant organellar signaling – from algae to higher plants. Primošten, Croatia. August 31st to September 3rd 2011. Abstract of poster presentation.
- **Cuypers, A., Jozefczak, M., Keunen, E., Bohler, S., Remans, T. and Vangronsveld, J.** (2012) Phytoremediation of cadmium-contaminated soils. A central role for glutathione in overcoming the oxidative challenge. Plant Growth, Nutrition & Environment Interactions – Plant Abiotic Stress Tolerance II. Vienna, Austria. February 18th to 25th 2012. Abstract of poster presentation.
- **Jozefczak, M., Schat, H., Remans, T., Keunen, E., Vangronsveld, J. and Cuypers, A.** (2012) Glutathione-related responses to cadmium toxicity in *Arabidopsis thaliana*. Plant Growth, Nutrition & Environment Interactions – Plant Abiotic Stress Tolerance II. Vienna, Austria. February 18th to 25th 2012. Abstract of poster presentation.

- **Keunen, E., Remans, T., Jozefczak, M., Vangronsveld, J. and Cuypers, A.** (2012) Cadmium-induced oxidative stress and mitochondria: A kinetic study in *Arabidopsis thaliana*. Plant Growth, Nutrition & Environment Interactions – Plant Abiotic Stress Tolerance II. Vienna, Austria. February 18th to 25th 2012. Abstract of poster presentation.
- **Vercampt, H., Keunen, E., Jozefczak, M., Remans, T., Vangronsveld, J. and Cuypers, A.** (2012) Oxidative stress in environmental toxicity: Effects of herbicide use on crops. Plant Growth, Nutrition & Environment Interactions – Plant Abiotic Stress Tolerance II. Vienna, Austria. February 18th to 25th 2012. Abstract of poster presentation.
- **Keunen, E., Remans, T., Jozefczak, M., Vangronsveld, J. and Cuypers, A.** (2012) Cadmium-induced oxidative stress and mitochondria: A kinetic study in *Arabidopsis thaliana*. 9th International Phytotechnology Society Conference. Diepenbeek, Belgium. September 11th to 14th 2012. Abstract of oral and poster presentation.
- **Jozefczak, M., Keunen, E., Schat, H., Remans, T., Vangronsveld, J. and Cuypers, A.** (2012) Cadmium induces a fast phytochelatin response at the expense of glutathione in *Arabidopsis thaliana*. SysBioLux: Conference on Integrative Biology in Plants, Microorganisms and the Environment. Belvaux, Luxembourg. November 8th 2012. Abstract of oral presentation.
- **Keunen, E., Jozefczak, M., Remans, T., Vangronsveld, J. and Cuypers, A.** (2013) Alternative respiration as a primary defence mechanism during the mitochondrial oxidative challenge in cadmium-exposed *Arabidopsis thaliana*. 8th International Conference for Plant Mitochondrial Biology. Rosario, Santa Fe, Argentina. May 12th to 16th 2013. Abstract of poster presentation.
- **Jozefczak, M., Keunen, E., Schat, H., Bohler, S., Remans, T., Vangronsveld, J. and Cuypers, A.** (2013) Cadmium-treated *Arabidopsis thaliana* reveal a regulatory role of glutathione in superoxide dismutase. 11th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Warsaw, Poland. July 17th to 19th 2013. Abstract of poster presentation.
- **Keunen, E., Jozefczak, M., Remans, T., Vangronsveld, J. and Cuypers, A.** (2013) Alternative respiration as primary defence during cadmium-induced mitochondrial oxidative challenge in *Arabidopsis thaliana*. 11th International Conference on Reactive Oxygen and Nitrogen Species in Plants. Warsaw, Poland. July 17th to 19th 2013. Abstract of poster presentation.

- **Jozefczak, M., Keunen, E., Schat, H., Remans, T., Bohler, S., Vangronsveld, J. and Cuypers, A.** (2014) Glutathione deficient *Arabidopsis thaliana* roots activate alternative pathways when cadmium induces a fast phytochelatin response at the expense of glutathione. 9th International Workshop on Sulfur Metabolism in Plants: Molecular Physiology and Ecophysiology of Sulfur. Freiburg, Germany. April 14th to 17th 2014. Abstract of poster presentation.
- **Keunen, E., Remans, T., Vangronsveld, J. and Cuypers, A.** (2014) The mitochondrial alternative oxidase as a modulator of the oxidative challenge in cadmium-exposed *Arabidopsis thaliana*. 19th Plant Biology Europe FESPB/EPSO Congress. Dublin, Ireland. June 22nd to 26th 2014. Abstract of oral presentation.
- **Keunen, E., Remans, T., Vangronsveld, J. and Cuypers, A.** (2014) The mitochondrial alternative oxidase modulates the oxidative challenge during moderate Cd exposure in *Arabidopsis thaliana* leaves. Environment Workshop: Reactive oxygen and nitrogen species and environment: A new vision for 2020. Baeza, Spain. October 15th to 17th 2014. Abstract of oral and poster presentation.