Environmental and Experimental Botany 91 (2013) 63–73

Contents lists available at SciVerse ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

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*

Environmental Biology, Centre for Environmental Sciences, Hasselt University – Campus Diepenbeek, Agoralaan Building D, B-3590 Diepenbeek, Belgium

ARTICLE INFO

Article history:
Received 22 January 2013
Received in revised form 12 February 2013
Accepted 19 February 2013

Keywords:
Alternative respiration
Arabidopsis thaliana
Cadmium
Gene families
Mitochondria
Oxidative stress

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.

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 transfer 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 ($O_2^{\cdot -}$) 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,

Abbreviations: AOX, alternative oxidase; Cd, cadmium; ETC, electron transport chain; ND, alternative NAD(P)H dehydrogenase; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; UCP, uncoupling protein.

* Corresponding author. Tel.: +32 11268326; fax: +32 11268301.
E-mail address: ann.cuypers@uhasselt.be (A. Cuypers).

0098-8472/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.envexpbot.2013.02.008

Cvetkovska and Vanlerberghe (2012) established for the first time that a lack of AOX increases steady-state *in planta* mitochondrial $O_2^{\circ-}$ 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 biological organisation 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.

2. Materials and methods

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 (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) in sunlight. The PAR provided at the rosette level was 170 $\mu mol\ m^{-2}\ s^{-1}$. 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.

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.

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 ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$).

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 strand 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 2× 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 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.

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.

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).

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.

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.

3. Results

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

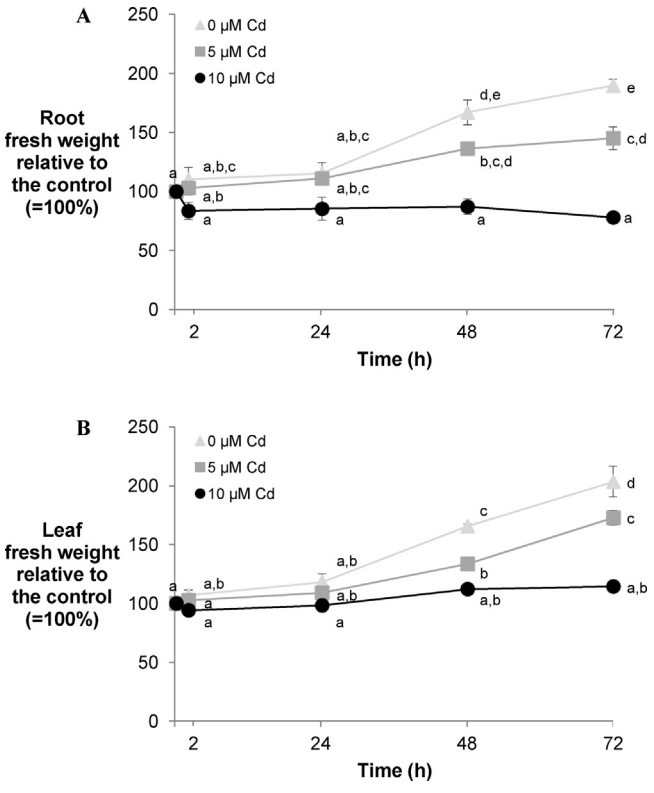

Fig. 1. Exposure to Cd has an effect on the growth of *A. thaliana* plants. Plants were exposed to 5 or 10 μM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 3 biological independent replicates relative to the control (0 h, 0 μM) set at 100%. (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 ($p < 0.05$).

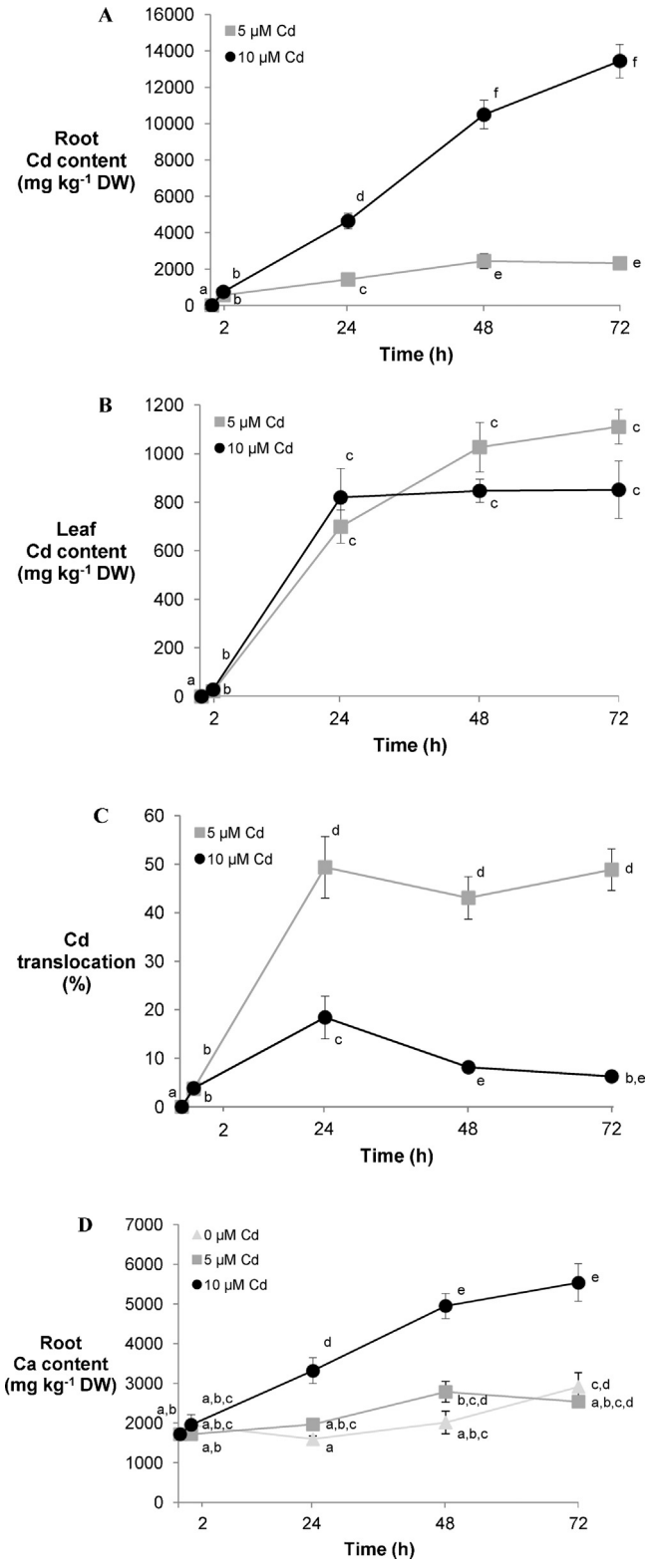

Fig. 2. Element concentrations in roots and leaves of *A. thaliana* plants exposed to 5 or 10 μM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of 3 biological independent replicates. (A) Cadmium content in mg kg^{-1} dry weight (DW) in the roots 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 ($p < 0.05$).

roots and leaves was determined at five harvest time points (Fig. 1). Cadmium obviously has a dose-dependent effect on *Arabidopsis* root growth, with complete inhibition in case of 10 μM Cd exposure (Fig. 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 (Fig. 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 (Fig. 1B), but a continuous increase for 5 μM Cd-exposed plants.

The uptake of Cd by the plants was verified by determining its content in dried roots and leaves (Fig. 2). The root Cd content not only increased over time, but also with the dose (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2D), which was not observed in the leaves. Alterations in the amounts of other nutrients were limited in both organs (data not shown).

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 (Fig. 3). In roots, exposure to 10 μM Cd significantly increased lipid peroxidation relative to the control at 72 h (Fig. 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 (Fig. 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, Fig. 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 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 1).

Table 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 *A. 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
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

Moreover, none of the other measured antioxidative genes were upregulated by Cd in the roots (Supplementary Table 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 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

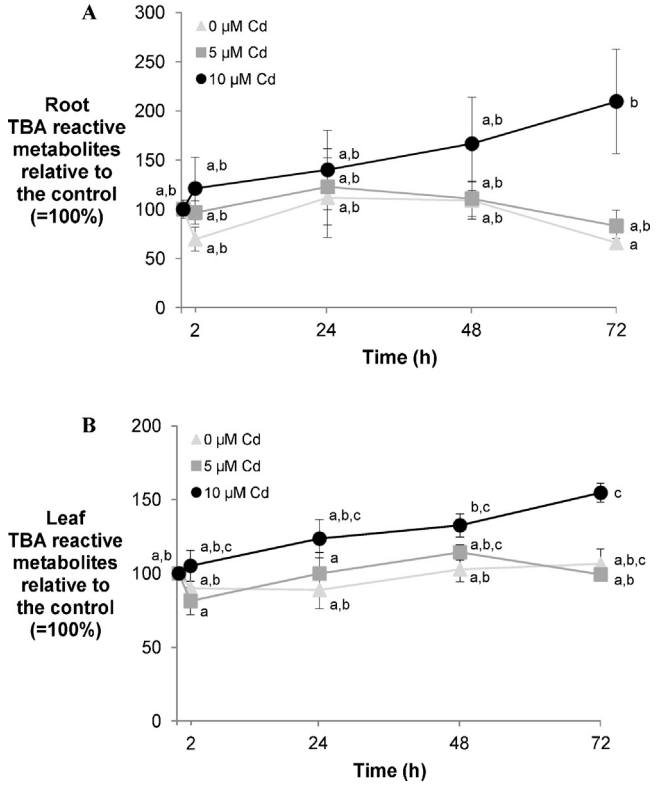

Fig. 3. Exposure to Cd leads to lipid peroxidation in roots and leaves of *A. thaliana* plants. Plants were exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean ± S.E. of at least 4 biological independent replicates relative to the control (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 ($p < 0.05$).

mitochondrial antioxidative enzymes were altered in a transient way (Supplementary Table 2). These results suggest the presence of alternative mechanisms to counteract the Cd-induced oxidative challenge at the mitochondrial level.

3.3. The response of alternative mitochondrial 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 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 3).

Transcriptional upregulation of the non-phosphorylating AOX, ND and UCP enzymes has shown to occur during various (a)biotic 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

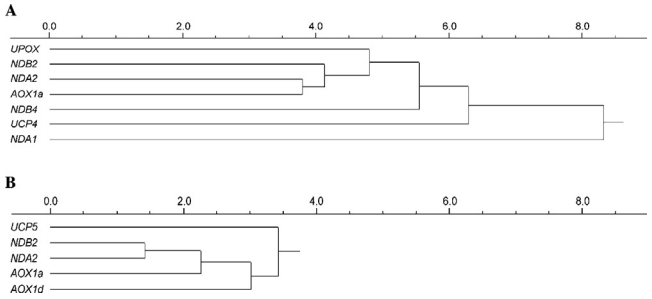

Fig. 4. GenEx clusters predicting possible co-regulation of genes encoding alternative respiratory pathway enzymes in roots (A) and leaves (B) of *A. thaliana* plants exposed to 5 or 10 µM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions.

in *AOX1c* expression levels (Supplementary Table 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 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 1).

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 4B). However, the expression of *NDA2*, *NDB2* and *NDB4* did show significant increases in both organs of Cd-exposed seedlings (Table 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 4B).

Several respiratory genes of the *AOX* and *ND* gene families displayed similar expression responses to Cd exposure (Table 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 (Fig. 4 A). Interestingly, these genes clustered together with *UPOX*, a marker for oxidative stress (Fig. 4A). In the leaves, *AOX1a* and *AOX1d* showed similarly regulated expression profiles with a cluster of *NDA2* and *NDB2* (Fig. 4B). Full clusters are shown in Supplementary Fig. 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 4C). In the leaves, exposure to both 5 and 10 µM Cd evoked a significantly reduced *UCP2*

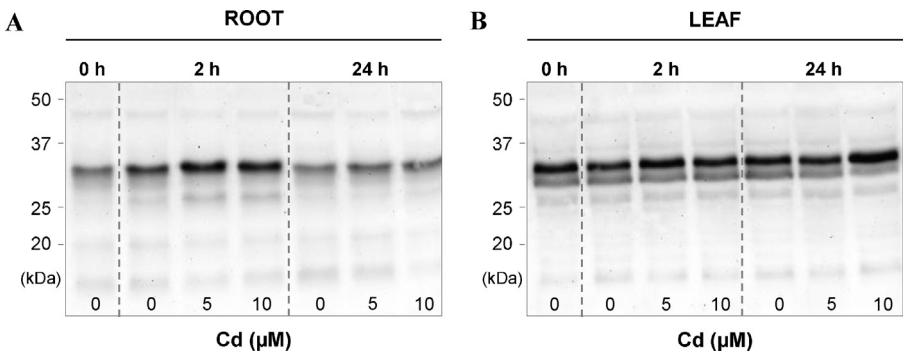

Fig. 5. Exposure to Cd leads to a fast but transient increase in AOX protein levels in roots and leaves of *A. thaliana* plants. Plants were exposed to 5 or 10 μM $CdSO_4$ during 2 and 24 h or grown under control conditions. (A) The amount of AOX protein detected via western blotting in the roots. (B)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

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 4C).

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 (Fig. 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 (Fig. 5B), which again disappeared at later time points (data not shown).

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 Fig. 6A. In addition, the dose-dependent increase of this isoform is demonstrated after 24, 48 and 72 h (Fig. 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 (Fig. 6B).

In control conditions, the external isoforms *NDB1* and *NDB2* dominate in the roots (Fig. 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 1, the abundance of this isoform remained rather low as compared to the others (Fig. 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 (Fig. 6D). Again, the relative abundance of *NDB4* was lower over the entire time course, although its transcripts highly increased as shown in Table 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 (Fig. 6E and F). In the roots, no specific isoform prevailed during Cd exposure (Fig. 6E), while the abundance of *UCP5* was prominent after exposure to both 5 and 10 μM Cd with a clear peak at 24 h (Fig. 6F).

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.

4.1. 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 (Fig. 1), without significant differences at prior time points as reported earlier (Cuypers et al., 2011). Elevated external Cd concentrations evoked dose-dependent increases in root Cd contents (Fig. 2A), which could be related to the inhibition of root growth in this condition (Fig. 1A). This dose-dependency was not observed in the leaves (Fig. 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 (Fig. 3). Corresponding to our data, Collin et al. (2008) reported only slightly augmented malondialdehyde levels in leaves of plants exposed to

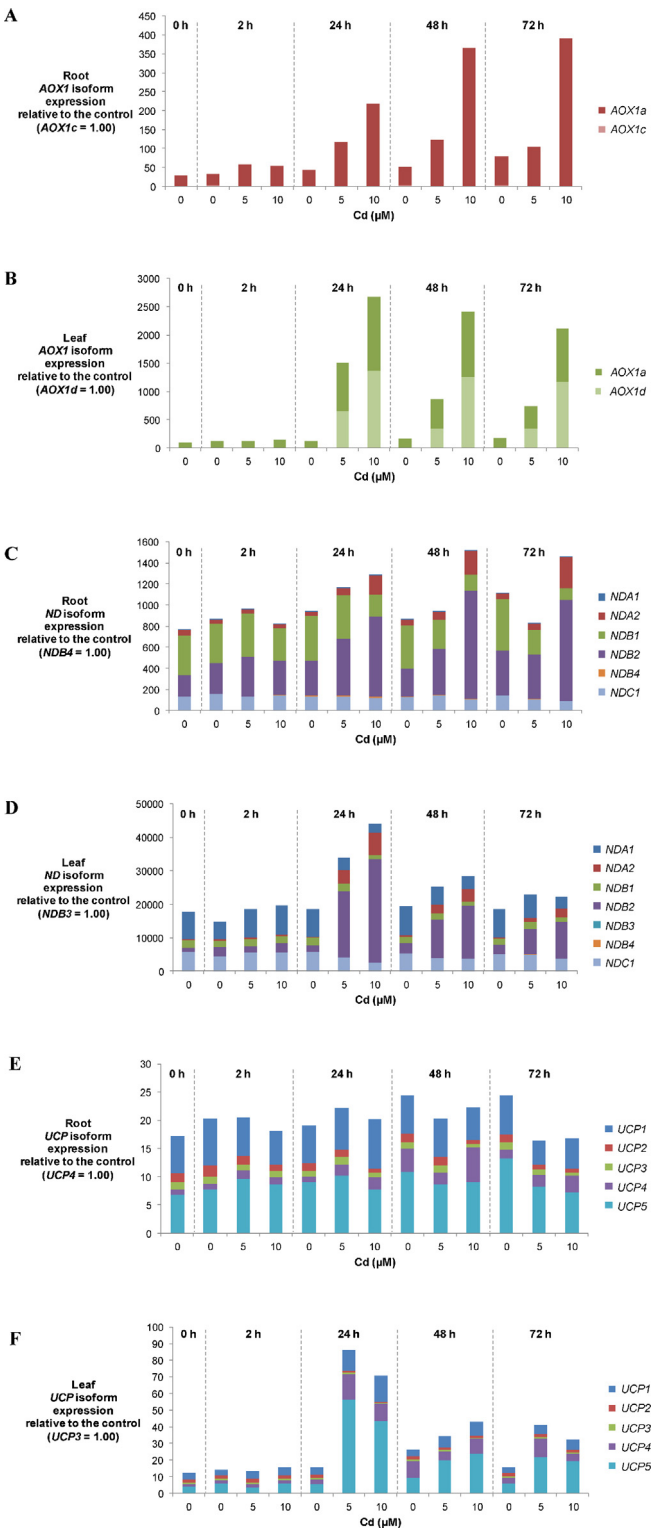

Fig. 6. Exposure to Cd has an effect on the relative abundance of *AOX, ND* and *UCP* gene family members in roots and leaves of *A. thaliana* plants. Plants were exposed to 5 or 10 μM $CdSO_4$ during 2, 24, 48 and 72 h or grown under control conditions. Data are given as the mean abundance of at least 4 biological independent 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.

75 μM $CdSO_4$ for even longer time periods as compared to our experimental setup.

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 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 $O_2^{\circ -}$ conversion to H_2O_2, its transcript levels did not change under Cd exposure in both roots and leaves (Table 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 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 wild-type 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 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 antioxidative enzymes are activated at the level of transcription.

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 1, Supplementary Table 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 (5 μM Cd) and leaves (5 and 10 μM Cd) (Fig. 6). The latter observation correlates well with the maximum Cd content reached at 24 h in the leaves (Fig. 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 (Fig. 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 3 and 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 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 (Fig. 6E and F), 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 (Fig. 6F) could therefore reflect the enhanced need to exchange respiration and 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 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 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. According to this, we observed a decreasing response for *NDB1* and *NDC1* transcription in both roots and leaves of Cd-exposed seedlings (Supplementary Table 4B). In addition, we demonstrated co-expression of specific *ND* and *AOX* isoforms in both roots (Fig. 4A) and leaves (Fig. 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 (Fig. 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 (Fig. 6C). In the leaves however, *NDA1* and *NDC1* prevailed (Fig. 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 (Fig. 6D), similarly to the response in roots (Fig. 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 (Fig. 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 1) were supported by augmented AOX protein contents in roots (at 2 h, Fig. 5A) and leaves (at 24 h, Fig. 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 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. 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.

Acknowledgments

This work was supported by the Research Foundation Flanders (FWO) by a PhD grant for Els Keunen and the project [G.0807.09]. Additional funding came from Hasselt University through BOF (Bijzonder Onderzoeksfonds) projects of Hasselt University [BOF08G01, BOF07N05] and the Methusalem project [08M03VGRJ]. We thank Carine Put and Ann Wijgaerts for their skilful technical assistance and gratefully acknowledge the advice by Professor Dr. Robert Carleer for the element determinations. We are also grateful to Dr. Thomas Elthon for the generous donation of the AOA antibody.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2013.02.008.

References

Andersen, C.L., Jensen, J.L., 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 Research 64, 5245–5250.

Bartoli, C.G., Gomez, F., Gergoff, G., Guiamét, J.J., Puntarulo, S., 2005. Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions. Journal of Experimental Botany 56, 1269–1276.

Bauwe, H., Hagemann, M., Fernie, A.R., 2010. Photorespiration: players, partners and origin. Trends in Plant Science 15, 330–336.

Bi, Y.H., Chen, W.L., Zhang, W.N., Zhou, Q., Yun, L.J., 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*. Biology of the Cell 101, 629–643.

Borecký, J., Nogueira, F.T.S., de Oliveira, K.A.P., Maia, I.G., Vercesi, A.E., 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. Journal of Experimental Botany 57, 849–864.

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

Chen, Z.H., Niki, E., 2011. Two faces of lipid peroxidation products: the 'Yin and Yang' principles of oxidative stress. Journal of Experimental and Integrative Medicine 1, 215–219.

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

Clifton, R., Millar, A.H., 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. Biochimica et Biophysica Acta 1757, 730–741.

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

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

Cuypers, A., Smeets, K., 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., Ravindran Nair, A., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J., 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., Vangronsveld, J., 2011. The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. Journal of Plant Physiology 168, 309–316.

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

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

DalCorso, G., Farinati, S., Maistri, S., Furini, A., 2008. How plants cope with cadmium: staking all on metabolism and gene expression. Journal of Integrative Plant Biology 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., Lin, H.H., 2010. Effects of cadmium stress on alternative oxidase and photosystem II in three wheat cultivars. Zeitschrift für Naturforschung C 65, 87–94.

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

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

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

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R., Van Breusegem, F., 2006. Transcriptomic footprints disclose specificity of reactive oxygen species signaling in Arabidopsis. Plant Physiology 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., Benavides, M.P., 2012. Unravelling cadmium toxicity and tolerance in plants: insights into regulatory mechanisms. Environmental and Experimental Botany 83, 33–46.

Garmash, E.V., Golovko, T.K., 2009. Effect of cadmium on growth and respiration of barley plants grown under two temperature regimes. Russian Journal of Plant Physiology 56, 343–347.

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

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

Keunen, E., Remans, T., Bohler, S., Vangronsveld, J., Cuypers, A., 2011a. Metal-induced oxidative stress and plant mitochondria. International Journal of Molecular Sciences 12, 6894–6918.

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

Krznaric, E., Verbruggen, N., Wevers, J.H.L., Carleer, R., Vangronsveld, J., Colpaert, J.V., 2009. Cd-tolerant *Suillus luteus*: a fungal insurance for pines exposed to Cd. Environmental Pollution 157, 1581–1588.

Lagriffoul, A., Mocquot, B., Mench, M., 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., Sandalio, L.M., 2002. Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. Plant Physiology and Biochemistry 40, 813–820.

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

Maxwell, D.P., Wang, Y., McIntosh, L., 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proceedings of the National Academy of Sciences of the United States of America 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. Functional Plant Biology 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., Finkemeier, I., 2008. Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. Plant Physiology 147, 101–114.

Navrot, N., Rouhier, N., Gelhaye, E., Jacquot, J.P., 2007. Reactive oxygen species generation and antioxidant systems in plant mitochondria. Physiologia Plantarum 129, 185–195.

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

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

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

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

Pastore, D., Trono, D., Laus, M.N., Di Fonzo, N., Flagella, Z., 2007. Possible plant mitochondria involvement in cell adaptation to drought stress. A case study: durum wheat mitochondria. Journal of Experimental Botany 58, 195–210.

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

Rasmusson, A.G., Geisler, D.A., 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., Meyer, Y., 2005. AtNTRB is the major mitochondrial thioredoxin reductase in *Arabidopsis thaliana*. FEBS Letters 579, 337–342.

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J., 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., del Río, L.A., 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. Journal of Experimental Botany 52, 2115–2126.

Schwarzländer, M., Fricker, M.D., 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. Biochimica et Biophysica Acta 1787, 468–475.

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

Sharma, S.S., Dietz, K.J., 2009. The relationship between metal toxicity and cellular redox imbalance. Trends in Plant Science 14, 43–50.

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

Smeets, K., Ruytinx, J., Semane, B., Van Belleghem, F., Remans, T., Van Sanden, S., Vangronsveld, J., Cuypers, A., 2008b. Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. Environmental and Experimental Botany 63, 1–8.

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

Srivastava, A., Jaiswal, V.S., 1989. Biochemical changes in duck weed after cadmium treatment. Enhancement in senescence. Water, Air, and Soil Pollution 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., 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. Molecular Plant 2, 284–297.

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

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

Van Assche, F., Cardinaels, C., 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. Environmental Pollution 52, 103–115.

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

Vangronsveld, J., 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., Wang, J., 2009. Is the maintenance of homeostatic mitochondrial signaling during stress a physiological role for alternative oxidase? Physiologia Plantarum 137, 392–406.

Vercesi, A.E., Borecký, J., de Godoy, M.I., Arruda, P., Cuccovia, I.M., Chaimovich, H., 2006. Plant uncoupling mitochondrial proteins. Annual Review of Plant Biology 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., 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., 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 and Cell Physiology 51, 810–822.

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

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