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Original Article

NADPH oxidases differentially regulate ROS metabolism and nutrient uptake under cadmium toxicity

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ABSTRACT

The role of NADPH oxidases under cadmium (Cd) toxicity was studied using *Arabidopsis thaliana* mutants *AtrbohC*, *AtrbohD* and *AtrbohF*, which were grown under hydroponic conditions with 25 and 100 μM Cd for 1 and 5 days. Cadmium reduced the growth of leaves in WT, *AtrbohC* and *D*, but not in *AtrbohF*. A time-dependent increase in H_2O_2 and lipid peroxidation was observed in all genotypes, with *AtrbohC* showing the smallest increase. An opposite behaviour was observed with NO accumulation. Cadmium increased catalase activity in WT plants and decreased it in *Atrboh* mutants, while glutathione reductase and glycolate oxidase activities increased in *Atrboh* mutants, and superoxide dismutases were down-regulated in *AtrbohC*. The GSH/GSSG and ASA/DHA couples were also affected by the treatment, principally in *AtrbohC* and *AtrbohF*, respectively. Cadmium translocation to the leaves was severely reduced in *Atrboh* mutants after 1 day of treatment and even after 5 days in *AtrbohF*. Similar results were observed for S, P, Ca, Zn and Fe accumulation, while an opposite trend was observed for K accumulation, except in *AtrbohF*. Thus, under Cd stress, RBOHs differentially regulate ROS metabolism, redox homeostasis and nutrient balance and could be of potential interest in biotechnology for the phytoremediation of polluted soils.

Key-words: Antioxidants; ascorbate; cadmium; glutathione; NADPH oxidases; nitric oxide; nutrient imbalance; oxidative stress; reactive oxygen species.

INTRODUCTION

Cadmium (Cd) is toxic for plants, animals and humans. In plants, just a few micromoles of Cd in the root environment can cause toxicity, resulting in root tip damage, reduced photosynthesis, disturbances in antioxidant defences in all plant organs and growth inhibition (Sandalio *et al.* 2001). Cd interacts with the water balance (Barceló & Poschenrieder 1990; Sandalio *et al.* 2001) and also actively inhibits the stomatal opening (Obata *et al.* 1996; Sandalio *et al.* 2001). In *Thlaspi caerulescens* (Boominathan & Doran 2003) and *Brassica juncea* (Pietrini *et al.* 2003), Cd can be accumulated to substantial concentrations without any toxic effects. The Cd tolerance of these species involves a complex network of homeostatic mechanisms that control the uptake, accumulation, trafficking and detoxification of metals (Clemens 2006). Once uptake and translocation of Cd have occurred, a number of mechanisms are involved in regulating the concentrations of free Cd ions among different plant organelles and hence minimize the damage to metabolism (Clemens 2006). It has been suggested that the non-protein thiol (NPT), glutathione (GSH), plays an important role in detoxification of heavy metals in plants (Gupta *et al.* 2010). No Cd-specific transporter has been identified, and Cd seems to be transported via several classes of Ca^{2+}, Fe^{2+} and Zn^{2+} transporters, affecting their uptake and distribution in plants and therefore inducing deficiency of those elements (Clemens 2006).

Different approaches have demonstrated that oxidative stress is one of the primary effects of Cd exposure, although the sources of reactive oxygen species (ROS) involved are not well established. Although the oxidation state of Cd does not change and therefore does not participate directly in Fenton-type reactions, Cd is assumed to cause the indirect formation of ROS by inhibiting antioxidant enzymes, impairing the respiratory chain or by displacing copper and iron ions from metalloenzymes and interfering with the redox status of cells (Valko *et al.* 2005; Sandalio *et al.* 2012). ROS are by-products of aerobic metabolism and are inevitably generated by a number of metabolic pathways and electron transport chains. ROS are partially reduced forms of molecular oxygen (O_2) and typically result from the transfer of one, two or three electrons to O_2 to form a superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), respectively, or an excited form of oxygen (singlet oxygen) (Halliwell & Gutteridge 2007). Different sources of ROS are associated

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with different organelles, plasma membranes, mitochondria, chloroplasts, peroxisomes and nuclei. NADPH oxidases (NOXs) have been shown to be one of the main sources of ROS associated with the plasma membrane and play many critical roles in plant development, plant defence responses, cell death, abiotic stress, stomatal closure and root hair development (Foreman *et al.* 2003; Yoshioka *et al.* 2003; Jones *et al.* 2007). NADPH oxidases (NOXs) are also called respiratory burst oxidase homologs (RBOHs) because of their homology with the NOX complex of mammalian phagocytes (Torres & Dangl 2005). *Arabidopsis* contains 10 homologous genes (*ATRBOH A-J*) (Torres & Dangle 2005). Another source of ROS is the glycolate oxidase (GOX), a key photorespiration enzyme associated with peroxisomes, amine oxidases and cell-wall-bound peroxidases. Under normal growth conditions, although the level of ROS production in the cell is low, stressful environmental conditions disrupt cellular homeostasis and trigger enhanced generation of ROS. Accumulation of ROS can be sensed as an 'alarm' signal, initiating defence responses that can lead either to stress acclimation or cell death, depending on the degree of oxidative stress experienced (Solomon *et al.* 1999).

Nitric oxide (NO) is a signalling molecule involved in a large number of physiological processes including germination, senescence, growth regulation, stomatal closure and pollen-pistil interaction (Besson-Bard *et al.* 2008; Serrano *et al.* 2015). Nitric oxide can be produced by non-enzymatic systems and enzymatic sources, including mainly nitrate reductase and L-arginine-dependent NO synthase-like activities (Neill *et al.* 2008; Gupta *et al.* 2011; Romero-Puertas *et al.* 2012). NO plays a key role in the regulation of cell responses to both biotic and abiotic stress (Neill *et al.* 2008; Mur *et al.* 2013). The role of NO in responses to Cd has been studied in depth in different plant species, and its production appears to be dependent on the dose and duration of treatment, with a NO burst occurring during a short period of treatment and a reduction in NO content after long exposure to the metal (Besson-Bard *et al.* 2009; Romero-Puertas *et al.* 2012; Pérez-Chaca *et al.* 2014).

The objectives of this study were to examine short-term and long-term (1 and 5d) responses of *Arabidopsis thaliana* to Cd and to analyse the main source of ROS under these conditions. The intracellular location of ROS production and the enzyme involved can regulate the specificity of cell responses to a specific stimulus (Mittler *et al.* 2011). This study therefore focuses on identifying the source of ROS induced by Cd by using different *Arabidopsis* mutants defective in NOXs *AtrbohC*, *AtrbohD* and *AtrbohF*. The effect of two different Cd concentrations in two different periods of treatment on WT and *Atrboh* plants was studied by analysing physiological and growth parameters, ROS and NO production, GOX, superoxide dismutase (SOD) and catalase (CAT) activity, glutathione and ascorbate metabolism and lipid peroxidation. Disturbances in the nutrient status of plants induced by Cd have been reported previously, although the regulation of these alterations is not yet well documented (Sandalio *et al.* 2012). Therefore, we have also studied the role of NOXs C, D and F on Cd and macro-nutrient and micro-nutrient content in roots and leaves.

MATERIALS AND METHODS

Plant material, growth conditions, treatments and growth analysis

Arabidopsis thaliana wild type (Col. 0) and mutant *AtrbohC, AtrbohD* and *AtrbohF* seeds were kindly supplied by Dr. Jonathan Jones (The Sainsbury Laboratory, Norwich, UK). Initially, the seeds were grown in compost/vermiculite (4:1) for three weeks. Healthy plants of equal size were then transferred to hydroponic cultures in plastic boxes containing 2 L of amended Hoagland medium, pH 6.2 (Hoagland & Arnon 1950). Plants were kept in a growth chamber at 22 ± 1 °C during a 16/8 light/dark cycle with 120–150 $\mu M\, m^{-2}\, s^{-1}$ of irradiance. Plants were treated with 25 and 100 μM $CdCl_2$, and those without metal were used as control. Cadmium-treated plants were harvested in a totally random manner after 1 and 5 days of treatment, and the leaves and roots were separated. Subsequently, growth and biochemical parameters were determined. Plant biomass was measured on a fresh weight basis.

Element estimation

The roots were rinsed twice for 5 min with cold MilliQ water to eliminate additional metal attached to the root surface, and both roots and shoots were oven-dried at 60 °C for 72 h. The dry plant tissues (50 mg) were ground and digested with 5 mL of concentrated HNO_3 using an open digestion system with a Velp Scientifica heating block (Milano, Italy). Heating was set at 130 °C for 2 h. Plastic caps were fitted to the vessels to prevent loss caused by volatilization. After digestion, the material was diluted five times with double distilled water. The concentrations of Cd and other microelements and macroelements were determined by inductively coupled plasma–optical emission spectrometry (ICP-OES) using a Perkin Elmer Optima 4300 DV (Shelton, USA) equipped with a cyclonic spray chamber and a concentric nebulizer. Standard solutions from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) were used as reference materials for Cd as well as all macro/micro element determinations.

Determination of hydrogen peroxide

Hydrogen peroxide (H_2O_2) was determined by fluorimetry basically as described by Romero-Puertas *et al.* (2004). Leaf tissues were extracted in 25 mM H_2SO_4 (1:2 w/v) in a mortar and centrifuged at 17 000 *g* for 25 min at 4 °C. Pigments were removed with activated charcoal and again centrifuged to 17 000 *g* for 25 min at 4 °C. H_2O_2 content was analysed by using homovanillic acid (excitation of 315 nm; emission of 425 nm) and horseradish peroxidase in 50 mM Hepes, pH 7.5 (Romero-Puertas *et al.* 2004), and the H_2O_2 concentration was obtained by using a standard curve of commercial H_2O_2.

Determination of nitric oxide

NO was analysed by fluorimetry using DAF-2DA as described by Rodríguez-Serrano *et al.* (2009). Extracts were obtained in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 0.2% (v/v) Triton X-100, 2 mM DTT and 1 mM PMSF. The homogenate was centrifuged at 17 000 g for 30 min at 4 °C, and the supernatants were collected and used for NO determination. The supernatant (100 μL) was incubated with 2 μL DAF-2DA (5 mM) in Hepes-KOH 50 mM buffer, pH 7.5, for 2 h at 37 °C. NO was then measured by analysing DAF-2-DA fluorescence (Ext. 495; Ems. 515 nm).

Analyses of ascorbate and glutathione

Leaf samples (200 mg FW) were ground in a cold mortar with liquid nitrogen and homogenized with 400 μL of 25 mM H_2SO_4. The homogenate was centrifuged at 17 000 g for 30 min at 4 °C, and the supernatant was collected for the analysis of ascorbate and glutathione.

The analysis of ascorbate was based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid (Kampfenkel *et al.* 1995). Fe^{2+} forms a red complex with bipyridyl that absorbs at 550 nm. Reduced (ASA) and oxidized (DHA) ascorbate was analysed using bipyridyl as described by Kampfenkel *et al.* (1995). The oxidized form DHA was reduced to ASA by pre-incubating the sample with dithiothreitol (DTT). Excess DTT was then removed with N-ethylmaleimide (NEM), and total ascorbate (ASA + DHA) content was measured. DHA content was estimated on the basis of the difference between total ascorbate and ASA. A standard curve covering the range of 2.5–60 μM ASA was used.

GSH and GSSG content was determined as described by Griffith (1980) based on the oxidation of GSH by DTNB (5,5′-dithio-bis-nitrobenzoic acid) to obtain GSSG and TNB (5-thio-2-nitrobenzene). GSSG was reduced to GSH by the action of GR and NADPH. The rate of DTNB reduction was recorded at 412 nm for 2 min. The contents were calculated using a standard curve of commercial GSH and GSSG (0–50 μM). GSH content was calculated from the difference between the total glutathione and GSSG.

Lipid peroxidation analyses

Lipid peroxidation was estimated by analyzing malondyaldehyde (MDA) content following the method described by El-Moshaty *et al.* (1993). Fresh shoot samples were collected (0.1 g FW) and ground in 10 mL of 0.2 M citrate–phosphate buffer (pH 6.5) containing 0.5% Triton X-100 using a mortar and pestle. The homogenate was filtered using two paper layers and centrifuged for 15 min at 20 000 *g*. One microlitre of the supernatant fraction was added to an equal volume of 20% (w/v) TCA containing 0.5% (w/v) of thiobarbituric acid. The mixture was heated at 95 °C for 20 min, then quickly cooled in an ice bath for 15 min and centrifuged at 10,000 *g* for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the absorbance value at 600 nm. The lipid peroxides were expressed as $\mu M\,g^{-1}$ malondialdehyde using an extinction coefficient of $155\,L\,mM^{-1}\,cm^{-1}$.

Enzymatic analyses

Frozen leaf tissues (300 mg FW) were ground in a mortar with liquid nitrogen in 600 μL of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 0.2% (v/v) Triton X-100, 2 mM DTT and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 17 000 g for 30 min., and the supernatants were collected and used for different enzyme assays. SOD isoenzymes were individualized by native-PAGE on 12% acrylamide gels and were localized by the photochemical method (Beauchamp & Fridovich 1971). Catalase (CAT) activity was measured spectrophotometrically according to the method described by Aebi (1984) by monitoring the disappearance of H_2O_2 at 240 nm and 25 °C for 2 min ($\varepsilon = 39.58\,M^{-1}\,cm^{-1}$). Glutathione reductase (GR) activity was measured according to Edwards *et al.* (1990) by monitoring the rate of NADPH oxidation as the decrease in absorbance at 340 nm for 2 min at 25 °C ($\varepsilon = 6.22\,mM^{-1}\,cm^{-1}$). Glycolate oxidase (GOX) activity was assayed spectrophotometrically by measuring the glyoxylate phenylhydrazine complex formed ($\varepsilon = 1.7 \times 10^4\,M^{-1}\,cm^{-1}$; Kerr & Groves 1975).

RNA isolation and reverse transcription polymerase chain reaction

Total RNA was isolated from leaves by the acid guanidine thiocyanate phenol-chloroform method described by Chomczynski & Sacchi (1987) using the Trizol reagent kit according to the manufacturer's instructions. Two micrograms of total RNA from leaves was used as template for the reverse transcription (RT) reaction. It was added to a mixture containing 0.5 μg oligo$(dT)_{12–18}$ anchored as primer (Invitrogen), 1× RT buffer, 40 units of RNase and 200 units of SuperScript II reverse transcriptase (Invitrogen). The reaction was carried out at 42 °C for 50 min, followed by a 15 min step at 70 °C and then cooled to 4 °C.

Amplification of tubulin cDNA from *Arabidopsis* was chosen as a control. The oligonucleotides used in this study are shown in Supporting Information Table 1. cDNAs were amplified by PCR as follows: 1 mL of the cDNA produced (diluted 1:10 or 1:20) was added to 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 1.25 unit of Hot Master Taq polymerase (Eppendorf) and 0.4 mmol of each primer (Supporting Information Table 1) in a final volume of 20 μL. Reactions were carried out in a Master Cycler (Eppendorf). A first step of 3 min at 95 °C was followed by 30 cycles of 30 s at 95 °C, 20 to 40 s at annealing temperature (55–65 °C, depending on the gene) and 30 to 45 s at 72 °C, with a final extension of 10 min at 72 °C. Amplified PCR products were detected after electrophoresis on 1.5% agarose gels (Serva) stained with GelRed (Biotium). Quantification of the bands was performed using a Chemidoc system (Bio-Rad Laboratories) coupled with a high-sensitivity CCD camera. Band intensity

was expressed as relative absorbance units. Each cDNA band density was first normalized by dividing it by the density of the tubulin band in the same lane in order to compensate for the variations in the cDNA loading onto the gel. The relative increase or decrease in gene expression in the Cd-treated leaves was then calculated by dividing the normalized band density of the gene from the Cd-treated leaves by that of the same gene from the untreated (control) leaves (Marone *et al.* 2001).

Protein determination and statistical analysis

Proteins were determined by the method described by Bradford (1976) using bovine serum albumin (BSA) as standards. All data were subjected to the Tukey–Kramer Multiple Comparisons Test. Data from 1 and 5 day treatments were analysed separately. Principal component analysis was carried out using the InfoGen programme (Balzarini & Di Rienzo 2012).

RESULTS

Growth analysis under Cd stress in wild type and *Atrboh* plants

The treatment of *Arabidopsis* plants with Cd did not negatively affect root growth after 1 day of exposure except in *AtrbohC* which showed a slight but statistically significant fresh weight reduction with the highest Cd concentration (100 μM; Fig. 1a). However, in *AtrbohD* mutants, plant growth with 25 μM Cd for 1 day lead to an increase in root biomass (Fig. 1a). After 5 days of treatment with Cd, a significant reduction in root biomass was observed in WT and *Artboh* plants, with the highest reduction being detected in plants treated with 100 μM Cd (Fig. 1a). Leaf biomass was more affected than roots by Cd treatment. In WT plants, leaf fresh weight was reduced by 50% after 1 day exposure to 100 μM Cd, while the reduction in growth was less than in *AtrbohC* and *AtrbohD* WT plants (Fig. 1b). *AtrbohF* plants were less affected by the Cd treatment, and a slight increase was observed after 1 day of treatment with 25 μM Cd (Fig. 1b). A similar trend was observed after 5 days of Cd treatment, although the reduction in leaf fresh weight was higher in *AtrbohD*, with a slight reduction being observed in *AtrbohF* with 100 μM Cd (Fig. 1b).

Hydrogen peroxide, lipid peroxidation and nitric oxide concentrations during Cd treatment

Cadmium induced slight but significant changes in H_2O_2 concentration in leaves after 1 day of treatment with 100 μM Cd in WT and *AtrbohD* plants, although a higher 3-fold increase was observed in *AtrbohD* mutants with 100 μM Cd and after 5 days of treatment. *AtrbohF* plants displayed a similar pattern to WT, although with 25 μM Cd for 1 day, a small, but significant, increase was observed (Fig. 1c). It is worth noting that the highest basal levels of H_2O_2 were observed in *Atrboh* plants in the absence of treatment as compared with WT plants (Fig. 1c). However, the increase in H_2O_2 induced by the metal was smaller in *AtrbohC* and *AtrbohD* than in WT plants, except with respect to *AtrbohD* after 5 days of treatment with 100 μM Cd (3-fold increase). Analyses of MDA, used as oxidative damage marker, showed that Cd promotes a time-dependent increase in lipid peroxidation in the leaves of WT and *Atrboh* mutants, with the greatest damage being noted in *AtrbohD* after 5 days of treatment with 100 μM Cd (Fig. 1d). *AtrbohF* plants did not show statistically differences in MDA content after 1 day of treatment (Fig. 1d). These results are consistent with the H_2O_2 content observed in all genotypes (Fig. 1c). The analyses of NO by spectrofluorimetry using DAF-2DA displayed an opposite trend to that of H_2O_2 in response to Cd treatment, with a gradual reduction in NO occurring parallel to the increase in the metal concentration and duration of exposure (Fig. 1e). The trends in NO content changes were similar in WT and *Atrboh* mutants, although the basal level of NO content in the absence of metal treatment differed; thus, in *AtrbohC*, NO content was higher than in WT plants, while in *AtrbohD* and *F* NO content was lower than in WT. In *AtrbohF* plants, NO content was not altered by exposure to the metal, except after 1 day of treatment with 100 μM Cd (Fig. 1e).

Contribution of photorespiration to H_2O_2 production under Cd toxicity

Given the results obtained in H_2O_2 accumulation shown in Fig. 1c, and in order to clarify the sources of H_2O_2 induced by Cd, we analysed the activity of GOX, a key enzyme in photorespiration and one of the main sources of H_2O_2 under light in green tissues. This activity showed a slight increase in WT plants treated with 100 μM Cd but was considerably induced after 5 days of treatment, mainly in *AtrbohC* and, to a lesser extent, in *AtrbohF* and *D* (Fig. 2a). Analysis of GOX transcripts demonstrated an induction of *GOX3*, mainly in *AtrbohC* and *AtrbohD* (Fig. 2b), while *GOX1* and *GOX2* did not change significantly (data not shown). These results suggest that in plants defective in NOXs, GOX could be an important source of ROS under long-term Cd exposure conditions.

Effect of Cd on antioxidative defences

The analyses of ascorbate content showed a Cd-dependent increase in total ascorbate, which correlated with the Cd concentration and period of treatment in WT and *Atrboh* plants, although, in *AtrbohC* and *D*, changes observed with 25 and 100 μM Cd after 1 day of treatment were not statistically significant and, in *AtrbohF* with 25 μM Cd total ASA, did not differ significantly from the untreated plants (Supporting Information Table S2; Fig. 3a,b). It is interesting to note that total ASA content was statistically significantly higher in *Atrboh* mutants than in WT in the absence of treatment, with *AtrbohF* showing the largest increase (2-fold higher than in WT; Supporting Information Table S2). After 5 days of treatment, the highest content of total ASA was also detected in *AtrbohF*

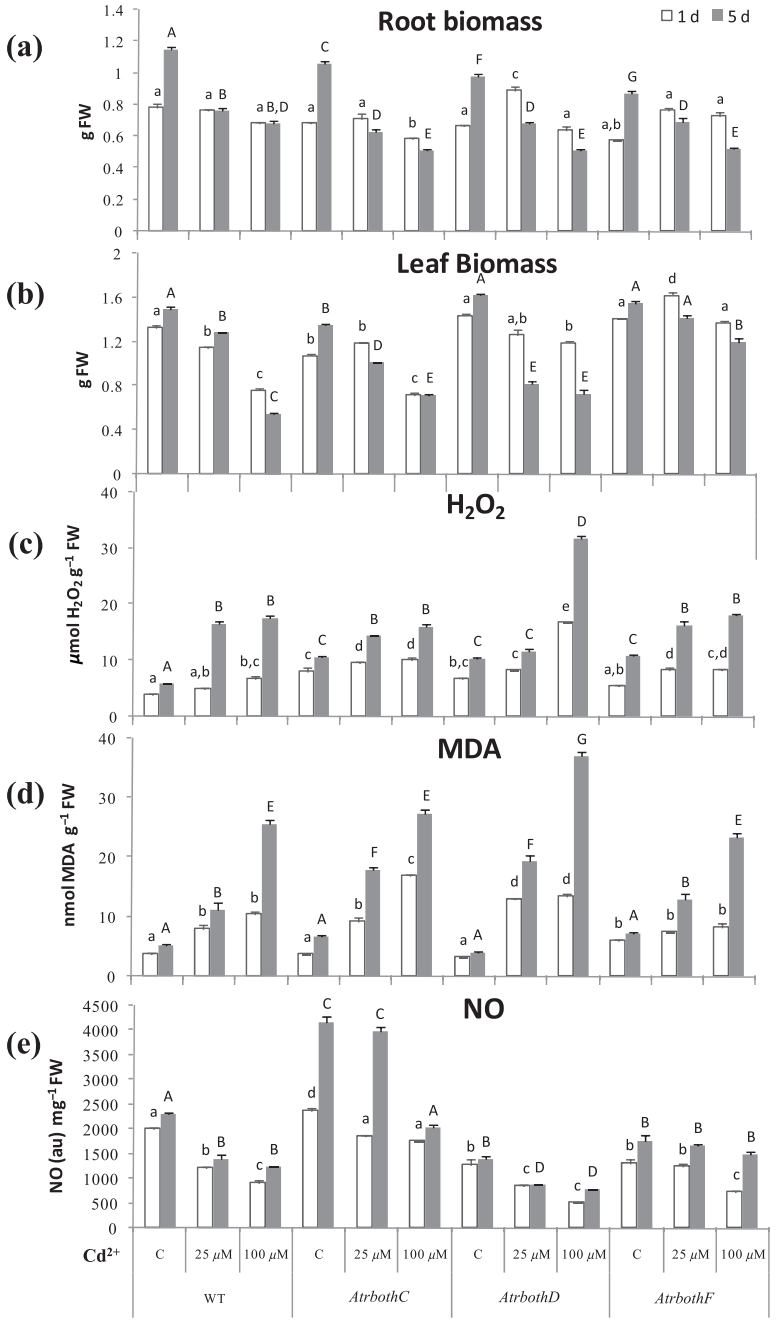

Figure 1. Cadmium effect on biomass, H_2O_2, lipid peroxidation and NO concentration. Changes on growth of roots (a) and shoots (b), hydrogen peroxide (H_2O_2) concentration (c), lipid peroxidation (MDA) (d) and nitric oxide (NO) concentration (e) in leaves from *Arabidopsis thaliana* WT and *Atrboh* plants grown in amended Hoagland and Arnon nutrient solution containing different concentrations of Cd (C: Control, 25 and 100 μM). Data are the mean of three independent replicates ± SD. Different letters denote statistically significance at $P < 0.05$ with the respective control for the same day.

(Supporting Information Table S2). However, the highest proportional increase in total ASA in response to Cd was observed in WT plants (5.2-fold higher), followed by *AtrbohC* (3.6-fold), *AtrbohD* (3.5-fold) and *AtrbohF* (2.3-fold) (Supporting Information Table S2). Treatment with Cd produced a dose-dependent and time-dependent increase in the reduced (ASA) and oxidized form (DHA) mainly in WT and *AtrbohF* plants (Fig. 3a,b). The ASA/DHA ratio decreased with the concentration of Cd in the nutrient solution in WT, *AtrbohC* and *AtrbohD* but increased in *AtrbohF* after 1 day of treatment (Fig. 3c; Supporting Information Table S2). After 5 days of treatment, the decrease in the ratio was smaller in all plants, except in *AtrbohF* which showed the highest Cd-dependent decrease (Fig. 3c; Supporting Information Table S2). It is worth noting that *AtrbohF* showed an opposite trend in the ASA/DHA ratio with respect to short and long periods of Cd exposure.

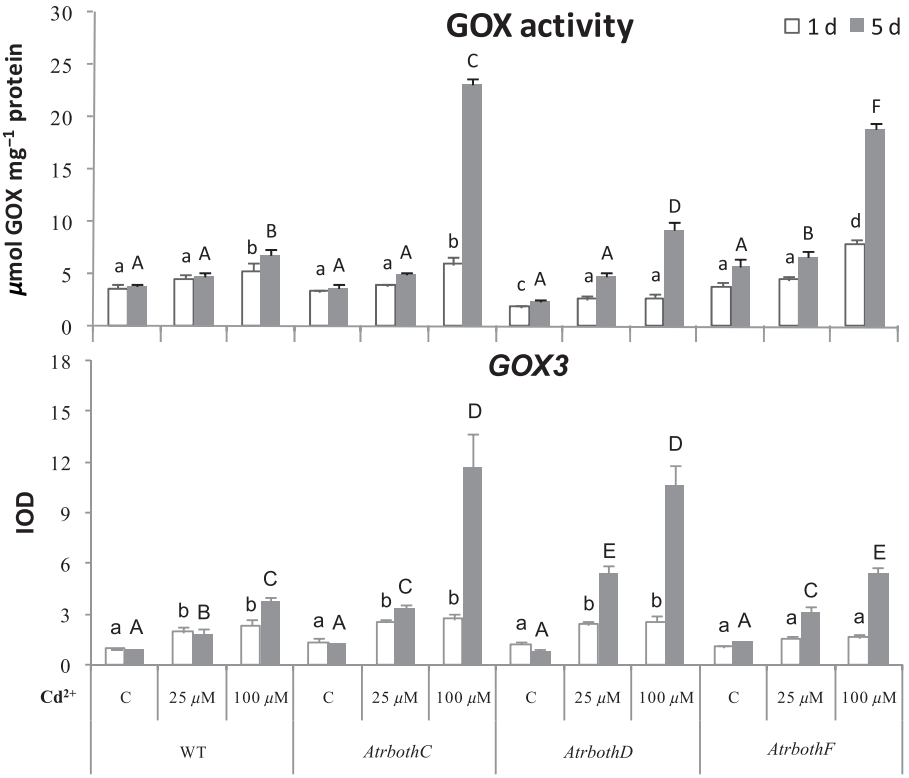

Figure 2. Cadmium effect on the activity and expression of GOX. WT, *AtrbohC*, *AtrbohD* and *AtrbohF* plants were grown for 1 day (a), and 5 days (b) with Cd (0, 25 and 100 μM Cd).GOX specific activity (a), and relative abundances of *GOX3* (b) transcripts at 1 day, and 5 days in *Arabidopsis thaliana* leaves exposed to 25 and 100 μM Cd or without Cd: control (C). Data were normalized considering the expression at WT(C) = 1. Means ± SD ($n = 4$). Different letters denote statistically significance at $P < 0.05$ with the respective control for the same day. IOD: integration of optical density.

Cadmium did not significantly alter total glutathione content after 1 day of treatment in WT, but significantly increased total GSH in *AtrbohD* and *F* with the highest Cd concentration (Supporting Information Table S2; Fig. 4a,b). The basal level of total GSH was higher in *AtrbohC* than in WT plants (Supporting Information Table S2). After 5 days of treatment, total GSH content significantly increased with 100 μM Cd in all the genotypes and also with 25 μM in *AtrbohF* (Supporting Information Table S2). The reduced form of GSH did not change significantly after 1 day of Cd treatment, except in *AtrbohF* grown with 100 μM Cd, and increased after 5 days of Cd treatment in *AtrbohD* and *AtrbohF* (Fig. 4a). The content of reduced GSH was significantly higher in *AtrbohC* and *D* than in WT in absence of Cd treatment (Fig. 4a). The content of the oxidized form (GSSG) increased with the period and concentration of Cd in *AtrbohC* and *AtrbohF*, and only significant changes were observed in WT plants after 5 days of treatment, with an opposite trend being observed in *AtrbohD* (Fig. 4b). The GSH/GSSG ratio was impaired by Cd after 1 day of treatment with 100 μM Cd in *AtrbohC*, in which a 3-fold reduction was observed with the highest Cd concentration (Fig. 4c, Supporting Information Table S2), while a Cd concentration-dependent increase was detected in *AtrbohD*, with no significant changes being noted in WT or *AtrbohF* plants (Fig. 4c; Supporting Information Table S1). After 5 days of treatment, a reduction in the redox ratio was observed mainly in WT plants, followed by *AtrbohC* and *AtrbohD,* while, in *AtrbohF*, it did not change significantly (Fig. 4c; Supporting Information Table S2).

Cadmium treatment also affected enzymatic antioxidative defences. CAT activity increased in parallel with the concentration of Cd and period of treatment in WT plants, while the opposite trend was observed in *Atrboh* mutants (Fig. 5a). However, it is interesting to note that the level of CAT activity in the absence of Cd treatment was higher in all *Atrboh* mutants than in WT plants (Fig. 5a). The analysis of the steady state level of *CAT1*, *CAT2* and *CAT3* transcripts by RT-PCR showed slightly differential expression levels in the three mutants depending on the concentration and period of treatment, with the greatest changes being observed in *CAT2* expression (Fig. 5c). However, *CAT3* and *CAT1* expression did not significantly change in the *Atrboh* mutants in comparison with WT plants or in response to Cd treatment, except in *AtrbohD* after 5 days of treatment (Fig. 5b,d). Concerning GR, a component of the ASA-GSH cycle involved in H_2O_2 removal, Cd treatment slightly induced the activity in WT plants, although the largest increase was observed in *Atrboh* plants after 5 days of treatment with 100 μM Cd, mainly in *AtrbohC* (5-fold) (Fig. 6a). Analysis of *GR1* and *GR2* transcripts, however, did not show any statistically significant changes in either transcript in WT and *Atrboh* plants (Fig. 6b,c). Superoxide dismutase is involved in $O_2^{\cdot-}$ dismutation, giving rise to H_2O_2 and O_2. The analysis of the different SOD isoforms by native-

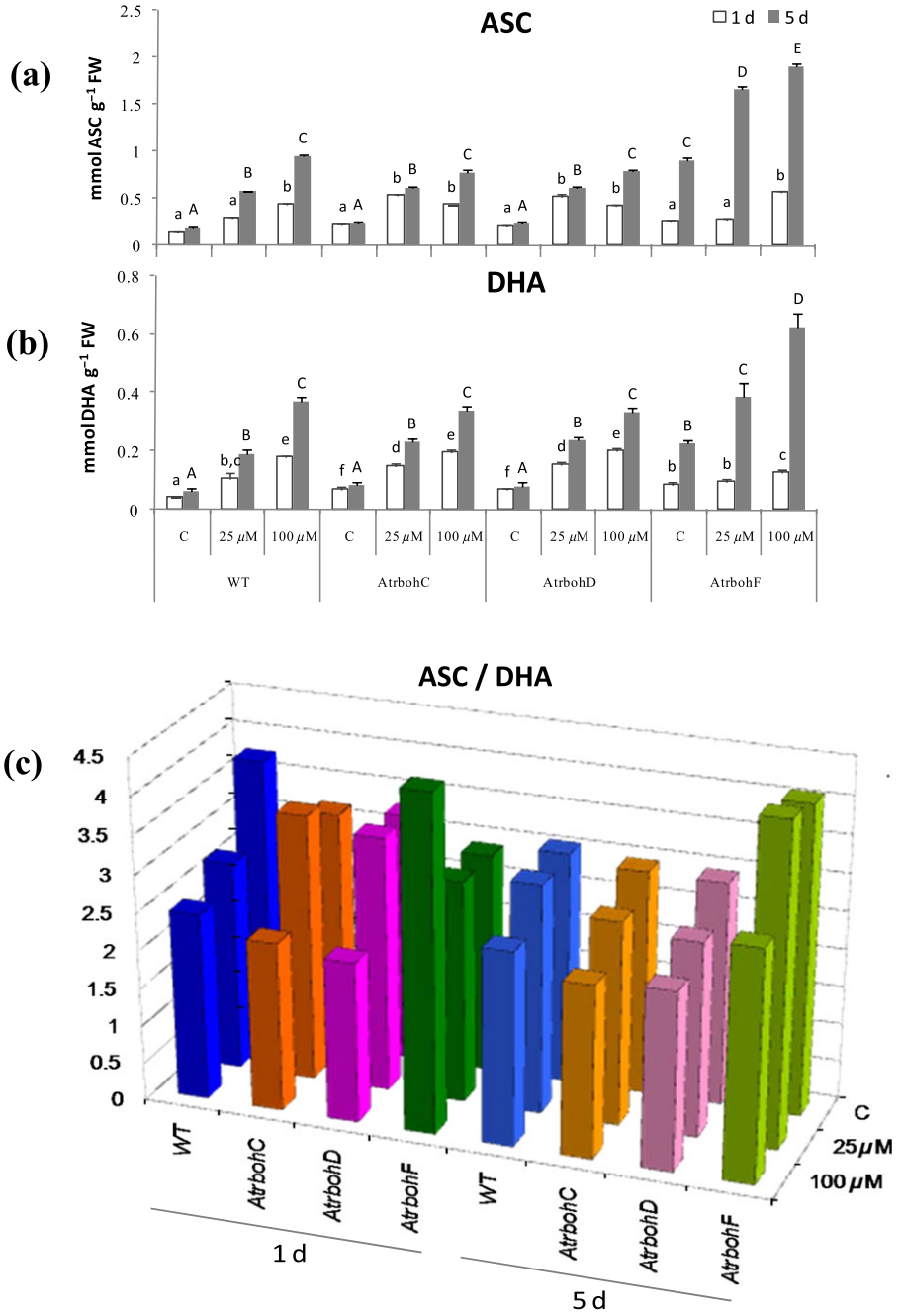

Figure 3. Disturbances in ascorbate metabolism induced by Cd treatment. Effect of Cd on the content of reduced ascorbic acid (ASA) (a), the oxidized form DHA (b) and the ASA/DHA ratio (c) in leaves from WT, *AtrbohC*, *AtrbohD* and *AtrbohF* Arabidopsis plants. Data are the mean of three independent replicates ± SD. Different letters indicate statistically significance at $P < 0.05$ with the respective control for the same day.

PAGE showed a sharp reduction in all SODs in *AtrbohC* plants as compared with WT plants under control conditions, while no apparent changes were observed in the other mutants (Fig. 7a,b). Treatment with Cd produced a reduction in Cu,Zn-SOD in WT and *AtrbohF* after 1 day of treatment with 100 μM Cd, while *AtrbohD* showed no change, with dose-dependent and time-dependent increases being observed in Mn-SOD and the Cu,Zn-SOD isoforms in *AtrbohC* (Fig. 7a,b).

Nutrient status under Cd stress

The analyses of Cd and other elements showed that Cd accumulation as well as macro/micronutrients uptake and translocation were disturbed by treatment with Cd and also by NOX deficiency in roots and leaves. Figure 8 shows metal and macronutrients accumulation after 1 and 5 days of Cd exposure. Cd content increases in roots in a dose-dependent and time-dependent manner. After 1 day of treatment, the highest

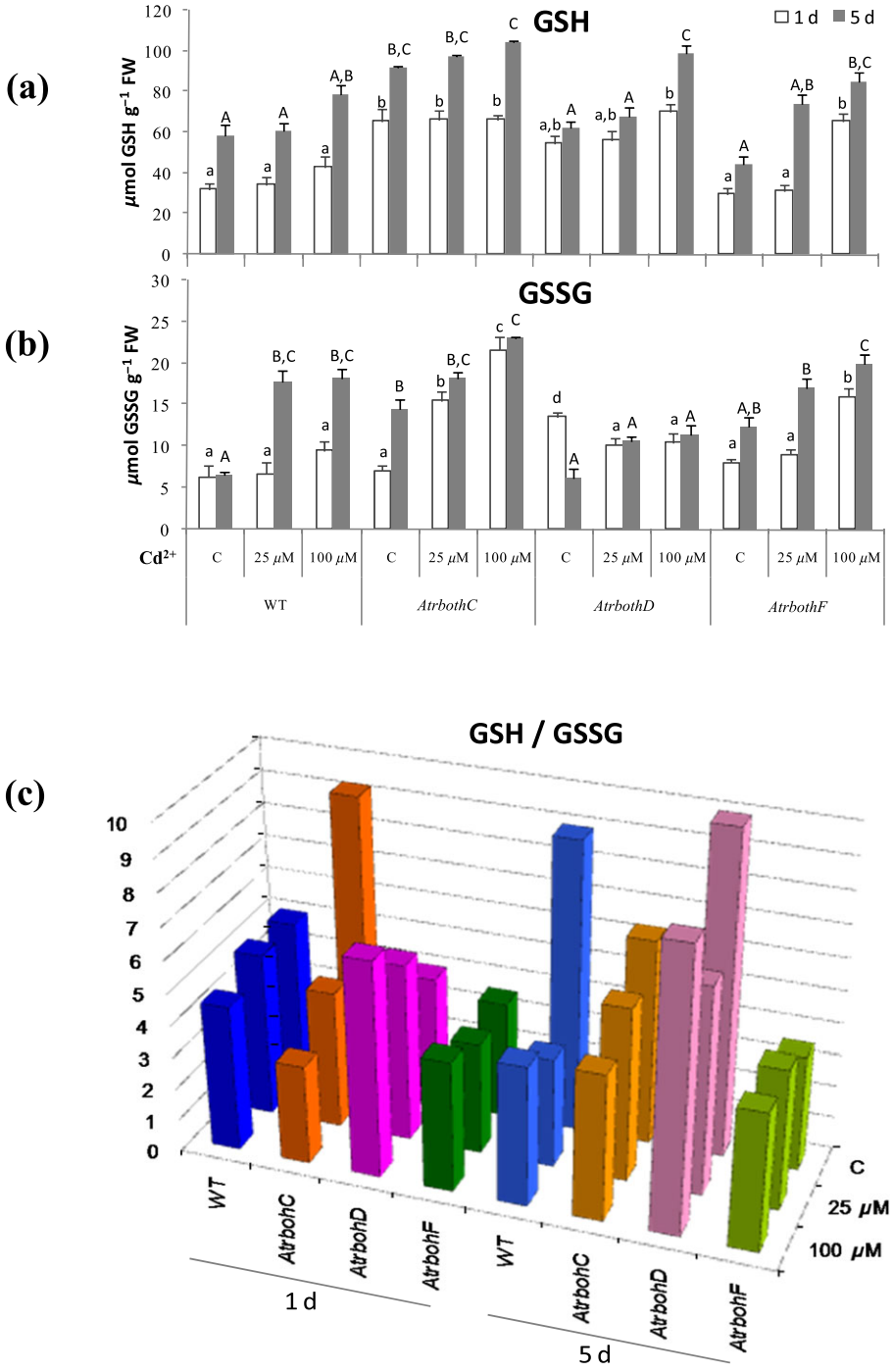

Figure 4. Cadmium affects glutathione content in leaves. Effect of Cd the reduced glutathione (GSH) (a), the oxidized form GSSG) (b) and the GSH/GSSG ratio (c) in leaves from WT, *AtrbohC*, *AtrbohD* and *AtrbohF* Arabidopsis plants. Data are the mean of three independent replicates ± SD. Different letters indicate statistically significance at $P < 0.05$ with the respective control for the same day.

concentration of Cd in roots was observed in *Atrboh* mutants, mainly in *AtrbohF* treated with 100 µM Cd (5-fold higher), followed by *AtrbohC*, *AtrbohD* and WT (Fig. 8a). An opposite trend was observed in leaves, in which the maximum Cd content was observed in WT plants treated with 100 µM Cd, while Cd accumulation levels in *AtrbohF* mutants were 6-fold lower than those in WT (Fig. 8a). Thus, WT plants are more efficient in relation to the translocation of Cd to leaves than *Atrboh* mutants. However, after 5 days of treatment, the trend in Cd accumulation was similar in the roots of both WT and *Atrboh* mutants, although Cd content was still slightly higher in *AtrbohF*, with Cd content in leaves being similar in WT, *AtrbohC* and *AtrbohD* and considerably lower (3-fold less) in *AtrbohF* (Fig. 8b). Cd severely disturbed the status of K in different ways in WT and *Atrboh* plants (Fig. 8c,d). K content increased in the roots and leaves of WT and *Atrboh* mutants

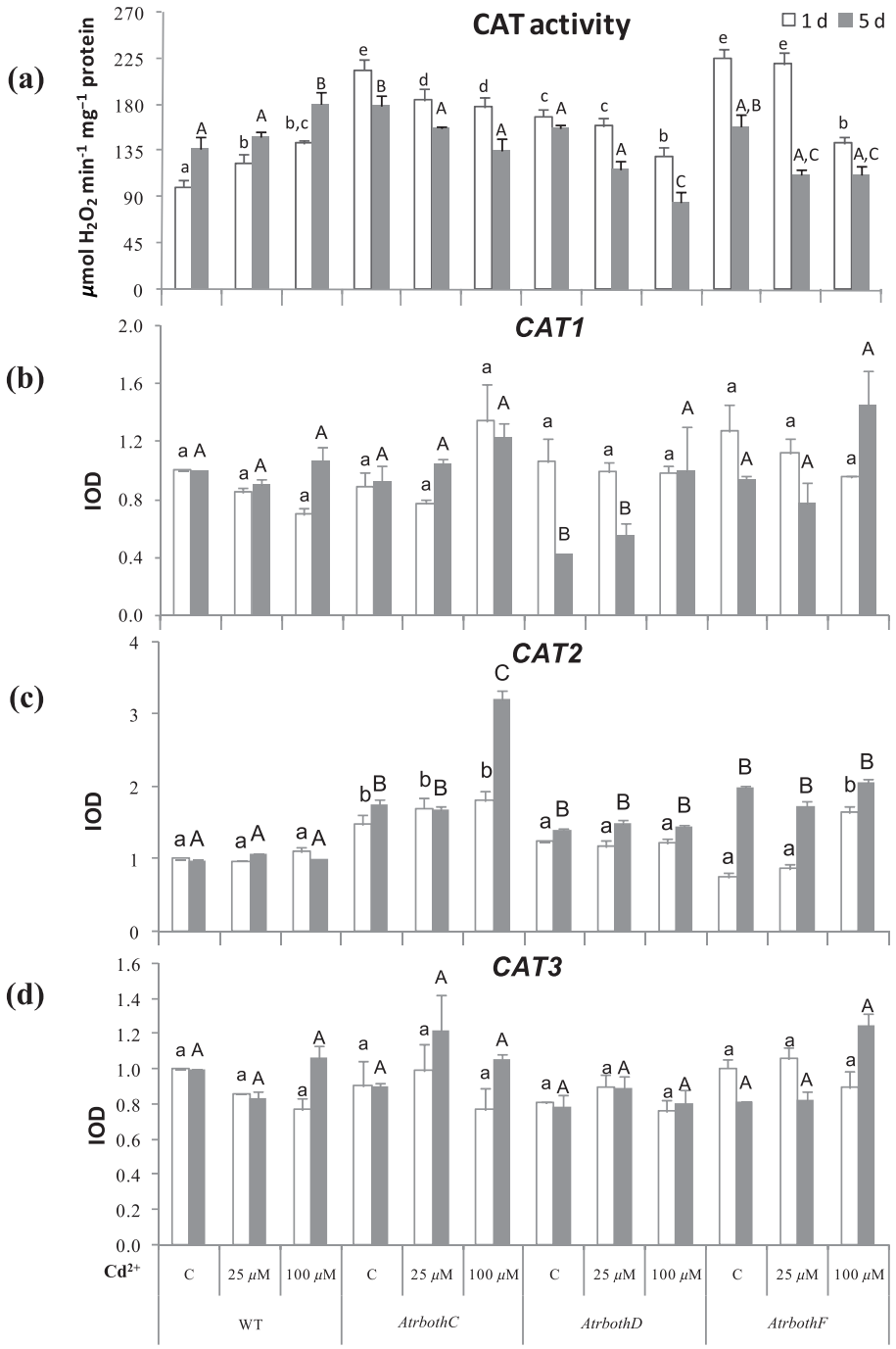

Figure 5. Effect of Cd treatment on CAT activity and expression. Catalase (CAT) specific activity (a), and relative abundances of *CAT1* (b), *CAT2* (c) and *CAT3* (d) transcripts were analysed in leaves from WT, *AtrbohC*, *AtrbohD* and *AtrbohF* after at 1 and 5 days of treatment with 25 and 100 µM Cd or without Cd: control (C). Data were normalized considering the expression at WT(C) = 1. Means ± SD ($n = 4$). Different letters were statistically significant at $P < 0.05$ with the respective control for the same day. IOD, integration of optical density.

with the concentration of Cd, mainly after 5 days of treatment, although the trend was different in *Atrboh* mutants (Fig. 8c). After 1 d of treatment, the highest accumulation of K took place in roots from *AtrbohF*, whose leaves also showed lowest K content, while *AtrbohC* and *AtrbohD* accumulated K mainly in leaves (Fig. 8c). After 5 days of treatment, WT and *AtrbohC* plants accumulate K mainly in roots, while *AtrbohD* and, to a lesser extent *AtrbohF*, translocated most K to leaves (Fig. 8c). In the case of sulphur (S), treatment with Cd reduced the content of this element in roots from *Atrboh,* and no significant changes were observed in WT after 1 d of treatment (Fig. 8e, f). However, S content decreased significantly in leaves from WT plants treated with 100 µM Cd, while no effect was observed in shoots from *Atrboh* mutants because of the Cd treatment. Interestingly, NOX deficiency gave rise to a 1.5-fold increase in S in roots and a 5 to 7-fold reduction in leaves in

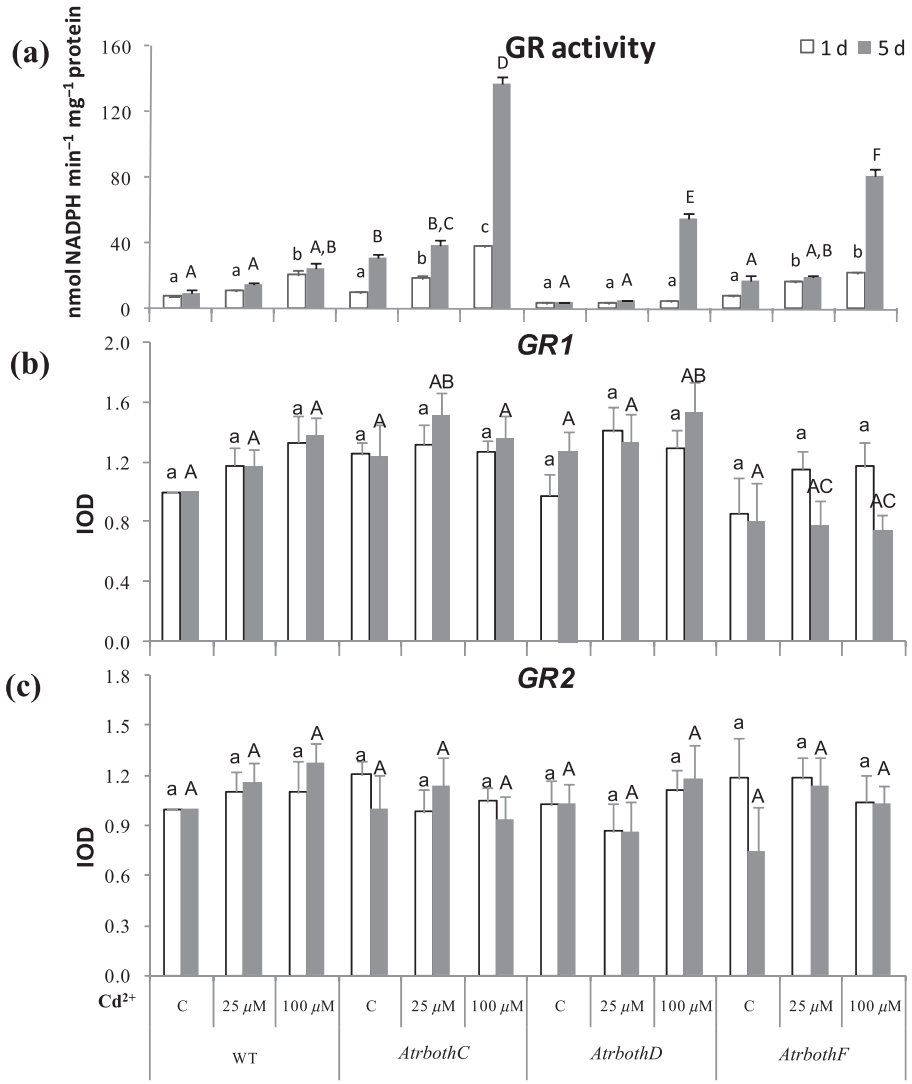

Figure 6. Effect of Cd treatment on the activity and expression of GR. Glutathione reductase (GR) specific activity (a), and relative abundances of *GR1* (b) and *GR2* (c) transcripts were analysed in leaves from WT, *AtrbohC*, *AtrbohD* and *AtrbohF* after 1 and 5 days of treatment with 25 and 100 μM Cd or without Cd: control (C). Data were normalized considering the expression at WT(C) = 1. Means ± SD (n = 4). Different letters were statistically significant at $P < 0.05$ with the respective control for the same day. IOD: integration of optical density.

control conditions and even a 15-fold reduction after 1 day of treatment, in comparison with WT plants, although these differences were less pronounced after 5 days of treatment (Fig. 8f). Cd induced a slight but insignificant increase in phosphorous (P) content in roots after 1 day of treatment in WT, *AtrbohD* and *AtrbohF*, while in *AtrbohC*, a statistically significant increase was observed in plants grown with 100 μM Cd (Fig. 8g). More significant changes were noted after 5 days of treatment in all plants, except *AtrbohC*, which recorded the highest P content in roots, although no statistical changes were observed with Cd treatment (Fig. 8h). P content increased considerably in leaves from WT and *AtrbohC* after 1 day of Cd treatment, while in *AtrbohC*, basal P content in the absence of Cd was 5-fold lower than in WT plants (Fig. 8g). In *AtrbohD* and *AtrbohF*, P content in leaves in the absence of Cd was similar to that in untreated *AtrbohC* but did not change significantly after 1 day of treatment with Cd (Fig. 8g). Thus, after 5 days of treatment, P content in leaves increased in a similar way in WT, *AtrbohD* and *AtrbohF*, with *AtrbohC* showing the highest content (Fig. 8h). Calcium (Ca) and Zinc (Zn) followed a similar pattern of changes to that observed in sulphur, with the reduction in the translocation of these elements to leaves in *Atrboh* mutants after 1 day of treatment being the most salient feature (Fig. 9a,c). After 5 days, the trend in Ca and Zn accumulation in roots and leaves was similar in WT and *Atrboh* mutants, showing a reduction in both elements in roots and mainly in leaves with the increase in Cd concentration, although these changes were statistically significant only in the roots of WT, *AtrbohC* and *F* and in the leaves of WT, *AtrbohC* and *F* for Zn (Fig. 9d). Concerning Ca, the reduction was significant in WT roots and leaves from all genotypes (Fig. 9b). Cadmium interferes with the uptake of other metals such as Fe and Cu. Cu content was reduced in roots and leaves in WT and *Atrboh* mutants, although differences were not statistically significant in

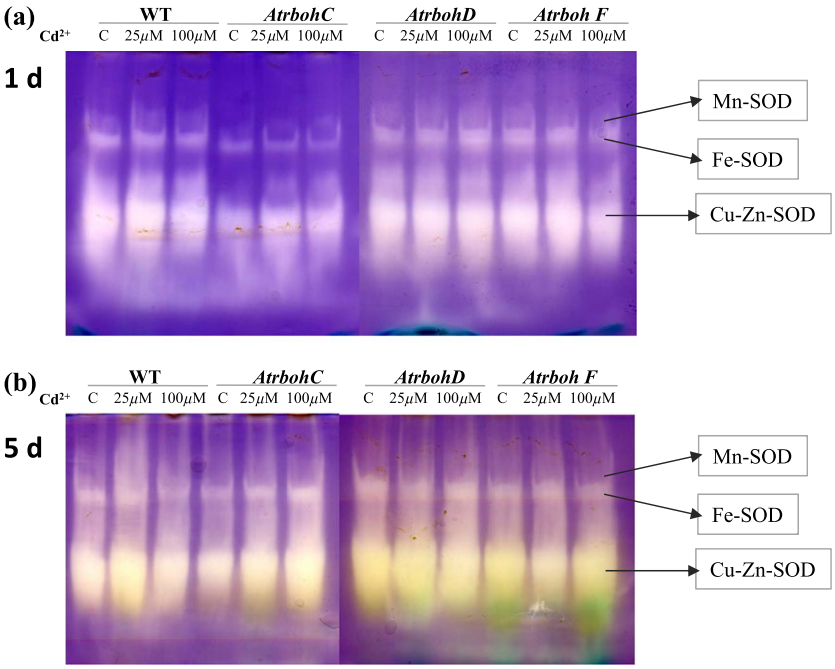

Figure 7. Effect of Cd treatment on SOD isozymes in leaves. WT, *AtrbohC*, *AtrbohD* and *AtrbohF* plants were grown for 1 day (a), and 5 days (b) with Cd (0, 25 and 100 μM Cd). SOD isoenzymes were individualised by native-PAGE (12% p/v polyacrylamide) and visualized using photochemical staining with NBT. Samples applied to the gels contained 80 μg of protein. This gel is representative of three different gels using different samples.

roots from *AtrbohD* and *AtrbohF* after 1 day of treatment or in *AtrbohC* and *AtrbohF* after 5 days of treatment (Fig. 9e,f). Iron content increased in roots and leaves from WT plants and varied in accordance with the concentration and period of Cd exposure, while in *Atrboh* mutants, Fe, whose concentration was severely reduced in leaves in comparison with WT plants did not change after 1 day of treatment in these green tissues and only increased significantly in roots (Fig. 9g,h). After 5 days of exposure, Fe content in leaves from *Atrboh* plants was similar to that in WT plants and increased with 100 μM Cd mainly in *AtrbohD* plants, while the content in roots was smaller than in WT plants, except in *AtrbohC* after treatment with 100 μM Cd (Fig. 9h).

Principal component analyses of ROS metabolism parameters and mineral content in WT and *Atrboh* mutants

In order to determine the differential patterns in WT plants and *Atrboh* mutants, PCA was conducted with respect to the Cd and nutrient concentrations, H_2O_2 and NO content, the ASA/DHA and GSH/GSSG redox couples and the activities GOX, GR and CAT. Comparison was carried out without taking into account the Cd concentration and time of treatment. This analysis showed two clear components (Fig. 10), PC1 showing differences between WT and *Atrboh* genotypes accounting for 39.6% of the variation and PC2 accounted for 30.9% of the variation and refers to differences between the three *Atrboh* lines. The main differences between WT and *Atrbohs* were associated to Zn, S and Ca in shoots, and Fe and Ca in roots. PC2 showed a higher similarity between *AtrbohC* and *AtrbohD*, mainly associated with Zn, P, S and Cu content in roots and K, Fe, P and Cd in shoots, and the content of H_2O_2, NO, the ratio GSH/GSSG and GR activity. However, *AtrbohF* was closer to WT with regard to Zn, S and Ca content in shoots and Fe and Ca content in roots. The main differences between *AtrbohF* and the other plants were associated with Cd and K content in roots, GOX activity and the ASA/DHA ratio. CAT activity followed the opposite patter in WT and *Atrboh* mutants, as shown before.

DISCUSSION

Biochemical and molecular analyses of gene regulation under Cd stress suggest that oxidative stress is one of the primary effects of Cd exposure (Smeets *et al.* 2009; Gallego *et al.* 2012; Romero-Puertas *et al.* 2012). NOXs have been suggested as an important source of ROS under Cd toxicity in cell cultures (Olmos *et al.* 2003), pea leaves (Romero-Puertas *et al.* 2004) and alfalfa roots (Ortega-Villasante *et al.* 2005). Analyses of the transcript levels of NOX also suggest that this protein plays a role in the oxidative stress imposed by Cd in *Arabidopsis* plants (Horemans *et al.* 2007; Smeets *et al.* 2009). In a previous study, Cd was observed to induce the expression of *RBOHC* and *RBOHD* in leaves and *RBOHF* in the roots of *Arabidopsis* plants exposed to low Cd concentrations (Smeets *et al.* 2009). In this study, we therefore analysed the contribution of ROS from RBOHC, RBOHD and RBOHF to Cd toxicity and plant responses

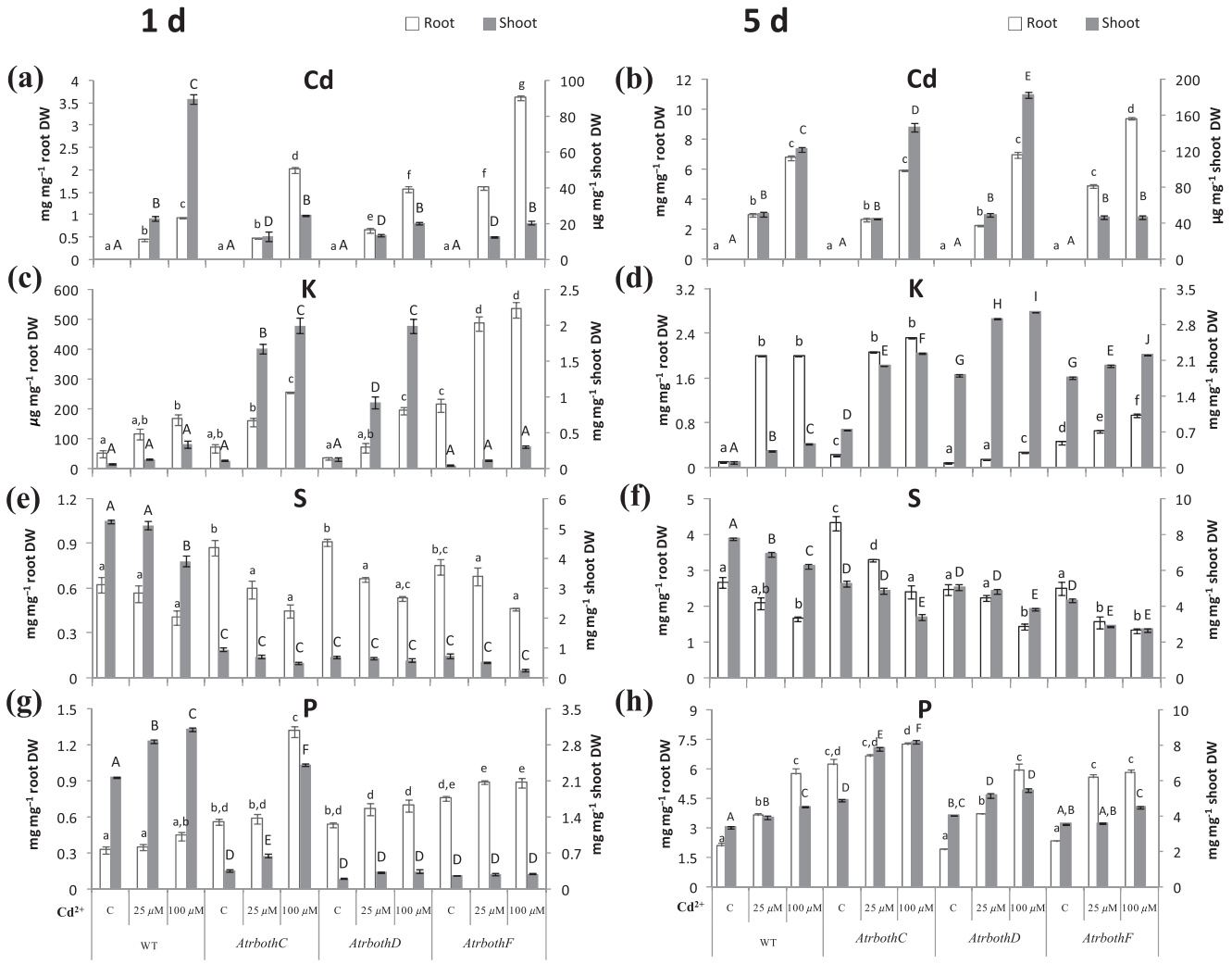

Figure 8. Cadmium effect on metal accumulation and macronutrient content in shoots and roots from WT and *Atrboh* plants. Cd concentration after 1 day (a), and 5 days of treatment (b), K concentration after 1 day (c) and 5 days of treatment (d); Sulfur (S) concentration after 1 day (e), and 5 days of treatment (f); phosphorous (P) concentration after 1 day (g), and 5 days of treatment (h). Data are the mean of three independent replicates ± SD. Different letters indicate statistically significance at $P < 0.05$ with the respective control for the same day.

to increasing concentrations of Cd and after short and long periods of treatment.

Differential effect of Cd on growth, ROS metabolism and nitric oxide accumulation in wild type and *Atrboh* plants

Inhibition of root elongation is one of the symptoms of Cd phytotoxicity (Schutzendubel *et al.* 2001; Sandalio *et al.* 2001; Gallego *et al.* 2012). However, in our study, Cd did not significantly affect root growth after 1 day of treatment neither in WT nor in *Atrbohs*, although a longer period of Cd exposure significantly reduced root fresh weight in all genotypes. Similar results were obtained when the effect of Cd on leaf fresh weight was analysed, although *AtrbohF* showed a higher tolerance to Cd toxicity. The tolerance of *AtrbohF* leaves to Cd could be explained by the low Cd concentration observed in this organ even in plants grown with 100 µM Cd.

The negative effect of Cd on leaves growth occurred in parallel to the increase in H_2O_2 content and oxidative damage to lipid in the leaves of wild type plants, while in *Atrboh* lines, differential responses among each other and with WT plants were observed. The analysis of H_2O_2 content in WT and *Atrboh* mutants suggests that each RBOH behaves differently from H_2O_2 production in response to Cd treatment, with RBOHC being most involved in H_2O_2 production under these conditions, while RBOHF does not contribute significantly to ROS production. In *Arabidopsis* plants, Remans *et al.* (2010) have shown that *RBOHs* are induced as key components in Cd-dependent H_2O_2 production, with *RBOHC* and *RBOHF* showing the highest induction levels in leaves. The lower contribution of RBOHF to ROS production observed in our studies could be because of the aforementioned lower Cd accumulation observed in leaves of these plants. The basal level of H_2O_2 in *AtrbohC* and *AtrbohD* was, however, higher than in WT plants, which suggests that disturbances in *RBOH* expression could have side effects in ROS metabolism that

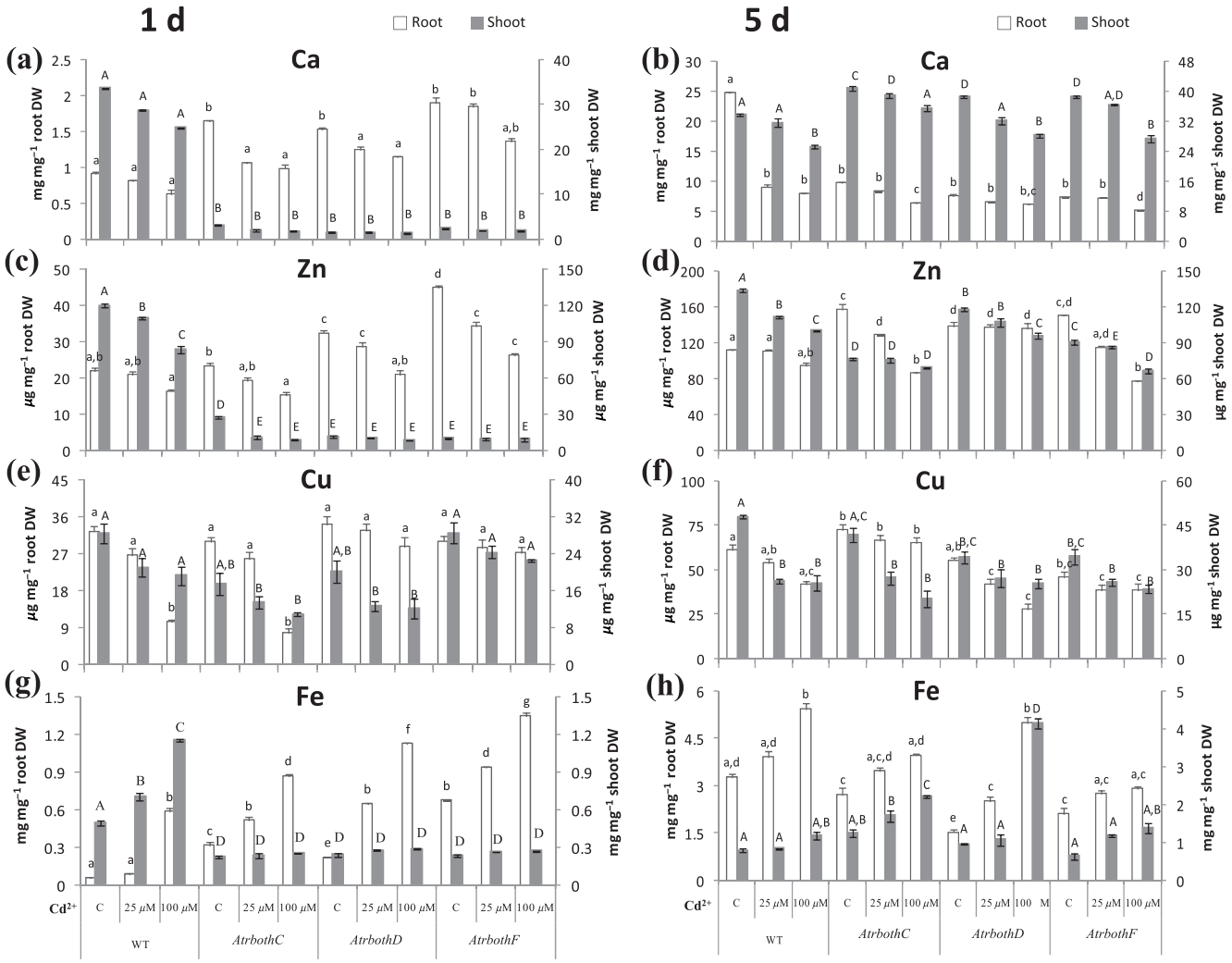

Figure 9. Cadmium effect on metal accumulation in root and shoot from WT and *Atrbohs* plants.Ca concentration after 1 day (a), and 5 days of treatment (b), Cu concentration after 1 day (c) and 5 days of treatment (d); Zn concentration after 1 day (e), and 5 days of treatment (f); Fe concentration after 1 day (g), and 5 days of treatment (h). Data are the mean of three independent replicates ± SD. Different letters indicate statistically significance at $P < 0.05$ with the respective control for the same day.

could affect other sources of ROS or antioxidative defences. Another two sources of ROS production are the peroxisomal GOX and the mitochondrial electron transport chain (Heyno *et al.* 2008; Romero-Puertas *et al.* 2012; Keunen *et al.* 2015). A relationship between NOX and GOX activity has been reported by Chaouch *et al.* (2012) in studies carried out on pathogenesis responses in *Arabidopsis* plants. Reciprocal crosstalk between ethylene, *RBOHC* and mitochondrial alternative oxidase (*AOX*) has recently been demonstrated in *A. thaliana* leaves during moderate Cd exposure (Keunen *et al.* 2015). The induction of GOX observed in this study could contribute to ROS production in response to Cd, mainly in the absence of RBOHs, which suggests a clear relationship between photorespiration and RBOHs after long periods of treatment. GOX activity has been reported to contribute to Cd-dependent ROS production in pea and soybean plants (Pérez-Chaca *et al.* 2014). Interestingly, *GOX3* which is associated with senescence, was up-regulated, which is in line with previous results demonstrating senescence induction by Cd (McCarthy *et al.* 2001).

Hydrogen peroxide homeostasis is regulated by CAT. CAT had a high reaction rate but lower affinity to H_2O_2, and therefore, it is more involved in H_2O_2 detoxification than in regulating H_2O_2 as a signalling molecule. In our study, CAT activity increased in response to Cd in WT plants, probably to cope with the increase of H_2O_2 production, while an opposite trend was observed in *Atrboh* lines. In addition, the basal level of CAT was higher in *Atrboh* lines than in WT plants, in line with H_2O_2 content (except in *AtrbohF* plants), which suggests that CAT can be regulated directly or indirectly by RBOHs. A similar increase in basal CAT activity has also been observed in *AtrbohC* plants previously (Gupta *et al.* 2013), suggesting a general cross relationship between RBOHs and CAT. These results and the changes observed in GOX activity suggest that there is a relationship between RBOHs and photorespiration. The analysis of CAT transcript, however, only showed

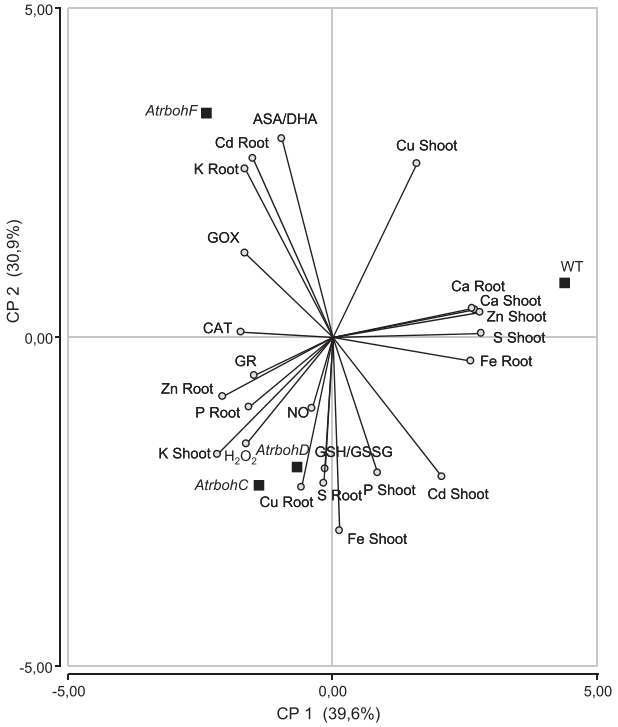

Figure 10. Graphical representations of principal component analysis (PCA) obtained from evaluation of mineral content, NO and ROS-related parameters in WT and *Atrboh* mutants.

upregulation of *CAT2* in *AtrbohC* and downregulation of *CAT1* in *AtrbohD* under severe stress conditions, and post-translational modifications in CAT, for instance, could regulate the activity. Thus, carbonylation and S-nitrosylation have been reported to inhibit the activity in pea plants exposed to Cd (Romero-Puertas *et al.* 2004; Ortega-Galisteo *et al.* 2012), while phosphorylation by the protein kinase PK8 has been observed to activate *CAT3* in *Arabidopsis* plants (Zou *et al.* 2015). In sunflower leaves, *CAT3* was upregulated in response to Cd (Azpilicueta *et al.* 2007).

The ascorbate-glutathione cycle, which is an important mechanism involved in controlling H_2O_2 levels in the cell, is present in all cell compartments. In this study, total ascorbate and total glutathione increased upon exposure to Cd in a dose-dependent and period of treatment-dependent way. Similar results have been observed in the same specie (Smeets *et al.* 2009; Skorzynska-Polit *et al.* 2003), although results can differ depending on the metal concentration used (Cuypers *et al.* 2011) and species (Gomes-Junior *et al.* 2006; Romero-Puertas *et al.* 2007; Pérez-Chaca *et al.* 2014). It is noteworthy that ASA content was slightly higher in *AtrbohC* and *AtrbohD* than in WT plants, and a similar increase in total ASA has, in fact, also been observed in *AtrbohC* previously (Gupta *et al.* 2013). In our study, *AtrbohF* showed the highest ASA content. This indicates that RBOH could play an important role in the regulation of ASA levels in the cell, although the mechanisms have not, so far, been established. GSH, in addition to participating in the ascorbate–glutathione cycle, is also involved in protection against heavy metals by acting as a chelator of metals, as a precursor of phytochelatins, and also regulates intracellular redox homeostasis and signalling processes (Gallego *et al.* 2012; He *et al.* 2015). In a similar way to ascorbate, total glutathione content was higher in the mutants, mainly in *AtrbohC*, suggesting a yet-to-be-established relationship between glutathione homeostasis and RBOH-derived ROS production. Glutathione biosynthesis is regulated by γ-glutamylcysteine synthetase (γ-ECS) activity and cysteine availability and *GSH1* and *GSH2* transcripts are induced by heavy metals (Schäfer *et al.* 1998). However, a previous study has shown that neither externally applied H_2O_2 nor intracellularly generated H_2O_2 lead to increased abundance of *GSH1* or *GSH2* transcripts in *Arabidopsis*, despite the increases in glutathione content under these conditions (Noctor *et al.* 2012). GSH biosynthesis is regulated by sulphur assimilation, and *Atrboh* mutants show higher levels of sulfur content under control conditions, although a decrease in both WT and *Atrboh* mutants was observed in response to Cd. This reduction could be because of the use of sulphate for GSH and phytochelatins synthesis (Clemens 2006). In the present study, Cd prompted changes in the GSH/GSSG ratio in WT and *Atrboh* lines, which were dependent on the time of treatment, except in *AtrbohF* which maintained the same value independently of dose and time of treatment. It has been suggested that the GSH/GSSG ratio may be involved in ROS perception (Shao *et al.* 2005; Li & Jin 2007) and could mediate the transcriptional regulation of genes involved in metal uptake and detoxification (Seth *et al.* 2012). However, the ASA/DHA rate followed an opposite trend in *AtrbochF* and the other genotypes. PCA analysis showed that the GSH/GSSG and ASA/DHA ratios can differentiate between the roles of RBOHs in the cell metabolism, showing a high similarity between RBOHC and D. Although the physiological implications of this are still unknown, it could be related to the different signatures for each RBOH. Therefore, RBOHs could play an important role in the redox homeostasis hub, showing a high degree of specialization that could be because of the differential expression of RBOHs in different types of cells.

Glutathione reductase plays a key role in the response to oxidative stress by maintaining the intracellular pool of GSH. Cd induced an increase in GR activity in both WT and *Atrboh* mutants, although the increase was much higher in *AtrbohC*, followed by *AtrbohF* and *AtrbohD* after a long period of treatment with the highest concentration of Cd. Our results are similar to those observed by Smeets *et al.* (2009) in *Arabidopsis*, in which *GR1* was upregulated, and by Pérez-Chaca *et al.* (2014) in soybean roots that showed upregulation of *GR5*. However, in this study the high activity observed after a long period of treatment in *Atrboh* mutants was not because of GR upregulation and could be explained by posttranscriptional modifications (carbonylation and *S*-nitrosylation; Bai *et al.* 2011). The parallel increase in GR and GOX observed in this study would suggest an induction of peroxisomal GR; in fact, in pea leaves, Cd did not affect total GR activity or *GR1* and *GR2* expression, while at subcellular level, an induction of peroxisomal activity was observed (Romero-Puertas *et al.* 2004).

Superoxide radicals are the primary ROS formed after electron transfer, which SOD converts into H_2O_2 and O_2. In multiple studies, diverse outcomes have been observed for SOD

activity in plants exposed to Cd, namely, an increase in wheat, a decrease in pea plants (DalCorso *et al.* 2008; Sandalio *et al.* 2001) and no significant effect on total SOD activity in *Arabidopsis* seedlings exposed to Cd (Smeets *et al.* 2009; Cuypers *et al.* 2011); however, downregulation of *CSD1* and *CSD2*, encoding CuZn-SOD 1 and 2, was observed in *Arabidopsis* leaves (Smeets *et al.* 2009). It is important to note that in our study, a different pattern was observed in SOD in *AtrbohC* mutants, which showed a considerably reduction in all isoforms. Similar results have been obtained with these mutants in studies carried out with arsenic (As) (Gupta *et al.* 2013). These results indicate that SODs could be regulated by RBOHC-dependent $O_2^{\cdot-}$, which is the main source of ROS under Cd toxicity in leaves, although as far as we know, the molecular mechanism has not yet been established and requires further study. However, RBOHC has been demonstrated that regulates the expression of two peroxidases under K deprivation in *AtrbohC* lines, although the mechanism needs to be elucidated in future studies (Shin & Schachtman 2004).

Nitric oxide is a very important signal molecule in the regulation of cell responses to biotic and abiotic stress conditions (Neill *et al.* 2008), and several authors have demonstrated the protective role of NO against the toxicity of Cd and other metals (Xiong *et al.* 2010; Sandalio *et al.* 2012; Liu *et al.* 2015). The production of NO in response to Cd has been widely studied, and contradictory results have been obtained (Groppa *et al.* 2008; Besson-Bard *et al.* 2009; Rodríguez-Serrano *et al.* 2009; Romero-Puertas *et al.* 2012; Pérez-Chaca *et al.* 2014). In this study all genotypes showed a dose-dependent reduction of NO, except in *AtrbohF*. However, in *AtrbohC*, NO content was highest in WT, and lower content being observed in *AtrbohD* and *F*, which is indicative of differential regulation of NO homeostasis by the different RBOHs. Similar results were observed by Gupta *et al.* (2013) in *AtrbohC* in studies carried out with As. He *et al.* (2013) have reported impaired NO generation and defective stomatal closure in response to UV-B in *AtrbohD* and *AtrbohF* mutants, which was rescued by exogenously applied NO, although no mechanistic explanation has been provided by these authors. NO can improve tolerance to heavy metal by regulating the general mechanisms of redox homeostasis in the cell, such as total GSH and ASA levels, regulating NOXs, and enzymatic antioxidants and also H^+-ATPase and H^+-PPase activities (Hossain *et al.* 2012). The regulation of NO content by RBOH and vice versa suggests the existence of feedback from ROS and NO homeostasis.

NADPH oxidases regulate Cd and nutrient uptake and translocation

Cadmium can easily enter plant roots and, in some plants, can be efficiently translocated to fruit (Clemens 2006). However, plant cells have no specific Cd transporters and metal uptake takes place through plasma membrane transporters involved in K, Ca, Mg, Fe, Zn, Mn and Cu incorporation (Clemens 2006). After entering the cytosol, Cd ions can bind to GSH and phytochelatins to prevent its toxicity (Clemens 2006).

In this study, we have found that Cd accumulation in roots from WT plants depends on the Cd concentration in the nutrient solution and the period of treatment and significant alteration were observed in *Atrboh* lines. Cadmium translocation to leaves after the first 24 h was also directly or indirectly regulated by RBOHs and was severely reduced in *Atrboh* mutant's shoots, which would explain the highest Cd accumulation in roots (2-fold higher in the presence of 100 μM Cd). However, after longer periods of exposure, Cd uptake and translocation escaped to regulation by *RBOHC* and *D*, while *RBOHF* continues to regulate its incorporation into roots and translocation to the leaf. These results suggest that NOX F plays an important role in regulating the uptake, chelation and translocation of Cd in *Arabidopsis* plants probably through ROS-mediated regulation of cation transporters. This could explain the less toxic effect of Cd observed in leaves from *AtrbohF* plants. Although this is the first study of the regulation of Cd uptake and translocation by RBOHs, we reported in a previous study that RBOHC could also regulate As uptake in *Arabidopsis* plants (Gupta *et al.* 2013). However, to determine whether this regulation takes place at the transcriptional level or through post-transcriptional modification it requires an in-depth study. The location of RBOHF in the xylem (Jiang *et al.* 2012) suggests that this protein plays an important role in the regulation of Cd translocation to the shoot.

The analyses of the effect of Cd on the distribution of different cations and macronutrients showed time-dependent and dose-dependent disturbances in all elements analysed in WT plants, and differential changes were also observed in *Atrboh* mutants. Cadmium induces a reduction in Ca, Cu and Zn in roots and leaves of WT plants, probably by competing for transporters such as Zinc transporter 1 (ZNT1s; Pence *et al.* 2000), $Calcium^{2+}$/$Hydrogen^+$ Antiporter, Cation/$Hydrogen^+$ Exchanger1 (*CAX1*, Baliardini *et al.* 2015), Natural Resistance Associated Macrophage Protein 1 (NRAMPs, Thomine *et al.* 2000; Clemens et al. 2006) and heavy-metal ATPases (HMAs; Hanikenne *et al.* 2008). Interestingly, the accumulation of these elements followed an opposite trend in *Atrboh* mutants, mainly after short periods of Cd treatment. Thus, RBOHs appear to regulate the translocation of Ca, Zn, Fe and Cd to the shoots during the first day of Cd treatment, with RBOHF being more specific to Cd, Zn and Fe. By contrast, in *AtrbohC* and *D* mutants, an increase in K translocation was observed in leaves. These results could be explained by the fact that ion channels and transporters are possible targets of ROS, although the mechanism involved is complex and can be regulated both transcriptionally and post-transcriptionally (Zepeda-Jazo *et al.* 2011; Rodrigo-Moreno *et al.* 2013). Thus, the suppression of the RBOHC in *Arabidopsis* roots prevents the up-regulation of genes induced by K deficiency, such as *KEA5* and *HAK5*, which is restored by exogenous application of H_2O_2 (Shin & Schachtman 2004). Interestingly, *HAK5*, is also regulated by nitrate, phosphate and Fe deficiency (Wang *et al.* 2002), suggesting a coordination and coregulation of these elements. Ma *et al.* (2012) have suggested that ROS produced by *AtrbohD* and *AtrbohF* activate the plasma membrane Ca-permeable channel, giving rise to an increase in cytoplasmic Ca levels, which mediates the modulation of Na/K homeostasis (Ma *et al.*

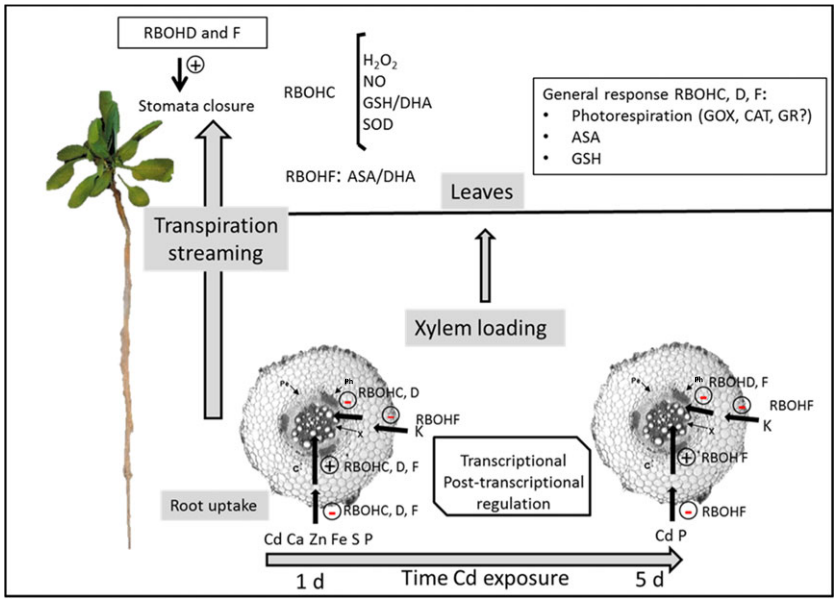

Figure 11. Diagram illustrating the role of RBOHs in the roots and shoots of *Arabidopsis* plants exposed to Cd stress. Uptake of Cd and other elements into the roots and xylem loading could be differentially regulated by RBOH C, D and F and by the period of Cd treatment. RBOH-dependent ROS could regulate ion transporters at the transcriptional or posttranscriptional level. Translocation of Cd and other ions to shoots can also be regulated by transpiration streaming which, in its turn, is regulated by RBOHD and F. In leaves, RBOHs regulate CAT, GOX and GR involved in photorespiration, as well the level of total ASC and GSH. RBOHC is the main source of ROS in leaves under Cd toxicity conditions, and also regulates NO accumulation, SOD expression and changes in the redox-couple GSH/GSSG ratio, while RBOHF regulates the redox-couple ASC/DHA ratio. Minus sign, repression; plus sign, activation; C, cortex; P, pericycle; Ph, phloem; X, xylem.

2012). RBOHF in *Arabidopsis* plants has been reported to be located in the root xylem, which increases ROS levels in response to salinity and stimulates *AtHKT1* expression and activity (Jiang *et al.* 2012). These results suggest that RBOHF plays an important role in regulating the root-to-shoot nutrient transport observed in this study. The translocation of Ca, Zn and Fe to leaves was inhibited to the same extent in *AtrbohC*, *D* and *F* after 1 day of treatment, while this process was not observed under longer Cd exposure periods. The differences observed with regard to the period of treatment could be explained by reprogramming of the plant to cope with disturbances in nutrient status. Our results also demonstrate the specific role played by RBOHF in Cd and K transport in roots, although the mechanism has not yet been established. However, some results suggest that transpiration stream plays an important role in this process. It has been observed that RBOHF in *Arabidopsis* plants can regulate Na transport by controlling transpiration in a process, in which NO from nitrate reductase is involved (Jiang *et al.* 2012). Thus, the effect observed on the nutrient imbalance of leaves in *Atrboh F* or *D* plants could be partly because of disturbances in the transpiration stream as a result of alterations in stomatal movements.

From our results, it can be concluded that Cd toxicity is associated with the induction of oxidative stress in the leaves of *A. thaliana* by elevated levels of H_2O_2 content, partly induced by NOXs. A scheme showing the contribution of each RBOH in response to Cd is shown in Fig. 11. We have demonstrated that RBOHs can differentially regulate H_2O_2 production, with RBOHC being the most important source of ROS under these conditions (Fig. 11); antioxidative defences are also differentially regulated, with SOD regulation by RBOHC as well as the regulation of redox-couple GSH/GSSG ratio by RBOHC and D and the ASA/DHA by RBOHF being the most important factors (Fig. 11). The regulation of CAT, GR and GOX is a characteristic response to Cd shared by all *Atrboh* genotypes. The differential responses observed in leaves from *Atrbohs* could be explained by the differential regulation of uptake and translocation of Cd and nutrients in the different *Atrbohs* (Fig. 11). The differential expression of RBOHs in different tissues and plant organs could partially explain the results obtained in this study. Thus, *RBOHD* and *RBOHF* have been demonstrated to be expressed throughout the plant, *RBOHA-C*, *G*, *E* and I are expressed mainly in roots and *RBOHH* and *J* are expressed in pollen (Sagi & Fluhr 2006). This fact and the differential contribution of each RBOH to the regulation of plant responses to Cd at different level, observed in this study, suggest a high degree of specialization, which can involve signatures from different RBOHs rather than the specific expression of any single one. This would contribute to the regulation of the complex network governing cell responses to metals. Our results also suggest that RBOHs can be important players in the nutrient regulatory hub and, for instance, be of potential interest in biotechnology to design new strategies to protect shoot cells from the damaging effects of an excess of some elements such as Cd.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Oligonucleotides used for RT-PCR analysis.

Table S2. Effect of Cd treatment on total ascorbate, ASA/DHA ratio, total glutathione and GSH/GSSG ratio in leaves from WT and *Atrboh* mutants.