Dehydroascorbate uptake is impaired in the early response of *Arabidopsis* plant cell cultures to cadmium

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Abstract

The balance between antioxidants, such as ascorbate (ASC) and glutathione, and oxidative reactive oxygen species (ROS) is known to play a pivotal role in the response of plant cells to abiotic stress. Here cell cultures of *Arabidopsis thaliana* were investigated with regard to their response to elevated levels of cadmium. At concentrations <100 μM, Cd induces a rapid and concentration-dependent H₂O₂ accumulation. This response could be inhibited by diphenylene iodonium (DPI, 20 μM). Reverse transcription-PCR analysis of three *RBOH* (respiratory burst oxidase homologues) genes showed an increased transcription of *RBOHF* after 15 min. No change in ASC concentration was observed during the first 3 h after Cd addition. In contrast, glutathione levels completely diminished within 1 h. This drop could be attributed to an increase in phytochelatin 4. At the plasma membrane, Cd further induced a significant decrease in dehydroascorbate (DHA) uptake activity (up to 90% inhibition after 4 h). This decrease is not present when cells are treated with LaCl₃ before exposure to CdCl₂. LaCl₃ is a typical inhibitor of Ca channels and prevents Cd uptake in these cells as well as the Cd-induced ROS production. Therefore, these results appear to indicate that Cd uptake is a prerequisite for the change in DHA transport activity. However, DPI did not prevent the drop in DHA uptake activity present in Cd-treated *Arabidopsis* cells, indicating that this response seems to be independent of the Cd-induced H₂O₂ production.

Key words: *Arabidopsis thaliana* cell cultures, ascorbate, cadmium, DHA uptake, glutathione, reactive oxygen species.

Introduction

Reactive oxygen species (ROS) have long been considered as detrimental molecules whose levels needed to be kept as low as possible. Recent literature has indicated differently, showing that ROS play an important role in the response of plants to biotic and abiotic stress, plant cell growth, programmed cell death (PCD), stomatal opening, hormone signalling, and regulation of gene expression. This multifaceted role for ROS indicates a tight control of their production and accumulation levels. The multiple functions exerted by ROS have been reviewed by a number of authors (Mittler, 2002; Overmyer et al., 2003; Apel and Hirt, 2004; Mittler et al., 2004; Foyer and Noctor, 2005). Metal stress, such as elevated levels of aluminium, cadmium and zinc, is known to be toxic to plants, triggering physiological responses (Sanità di Toppi et al., 1999), and specifically for Cd also genotoxic effects (Zhang and Xiao, 1998). It has become clear that at least part of the metal-induced phytotoxicity can be attributed to oxidative stress. An increased production of ROS has been reported after exposure to Cu (Raeymaekers et al.,

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Abbreviations: ASC, ascorbate; AAP, 4-aminoantipyrine; DCHBS, 3,5-dichloro-2-hydroxybenzenesulphonic acid; DHA, dehydroascorbate; DPI, diphenylene iodonium; GSH, glutathione; PCD, programmed cell death; RBOH, respiratory burst oxidase homologues; ROS, reactive oxygen species; UBQT 10, ubiquitin 10.

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2003) or Al (Yamamoto et al., 2002), and after exposure of plants or plant cell cultures to Cd stress (Piqueras et al., 1999; Dixit et al., 2001; Polle, 2001; Schützendübel et al., 2001; Olmos et al., 2003; Romero-Puertas et al., 2004; Cho and Seo, 2005). The biochemical mechanism of ROS production during metal stress is, however, still the subject of debate. Cd does not participate in redox reactions. Therefore, in contrast to essential transition metals such as Cu and Fe, Cd is unable to produce ROS through a Fenton-like reaction. Most studies point to an active ROS production induced by Cd catalysed by an NADPH oxidase-like enzyme in the plasma membrane (Piqueras et al., 1999; Olmos et al., 2003; Raeymaekers et al., 2003). However, other pathways including the activity of cell wall peroxidases or polyamine oxidases have also been suggested to contribute to H$_2$O$_2$ production (Apel and Hirt, 2004). Recently Garnier and co-workers (2006) showed that exposure of BY-2 tobacco cell cultures to millimolar concentrations of CdCl$_2$ resulted in three successive waves of ROS production from different origins and subcellular localizations. Finally, plants have an extensive antioxidant scavenging system consisting of low molecular weight antioxidants such as ascorbate (ASC) and glutathione (GSH) that, together with antioxidative enzymes, control the level of ROS both in non-stress and in oxidative stress conditions (Mittler et al., 2004). However, an imbalance in the dynamic equilibrium that in healthy cells exists between the formation of ROS and the activity of this antioxidant scavenging systems can also result in ROS accumulation (Schützendübel et al., 2001; Mittler et al., 2004).

The ability to synthesize GSH seems to be crucial in the protection of plants from Cd, as has been witnessed by a tight correlation between the GSH level and the level of tolerance of different plants (Schützendübel and Polle, 2002, and references therein). GSH is important not only as a ROS scavenger, but also as a base unit in phytochelatin production. Phytochelatins are polymers that can bind and thereby detoxify heavy metals (Zenk, 1996; Foyer and Remmenga, 2000; Nocito et al., 2002). As such, a rapid depletion of GSH was shown in Cd stress conditions (Schneider and Bergmann, 1995; Noctor et al., 1998; Schützendübel et al., 2001; Hsu and Kao, 2005). The maintenance of the cellular redox homeostasis is the result of a balance between the synthesis, consumption, and recycling of the most abundant antioxidants GSH and ASC. The role of enzymes of the so-called Halliwell–Asada cycle in the response of plant cells to Cd has often been studied (Schützendübel and Polle, 2002; Semane et al., 2007). However, as has been indicated above, the first ROS production seems to be extracellular, probably resulting first in a changed apoplastic redox status. The regeneration of apoplastic ASC and therefore the apoplastic redox status seems to be intimately linked to transport phenomena across the plasma membrane. In vitro studies showed that monodehydroascorbate (MDA), the first oxidation product of ASC, can be reduced to ASC with the aid of a trans-plasma membrane electron transport mediated by an ASC-reducible cytochrome b$_{561}$ (Horemans et al., 1994). On the other hand, the regeneration of ASC from its fully oxidized form, dehydroascorbate (DHA), was suggested to occur after uptake of DHA into the cells through a high affinity DHA carrier and internal reduction (Horemans et al., 1998). Interestingly, in animal differentiated HL-60 cells, it was shown that the phorbol ester PMA stimulates DHA uptake activity up to 10-fold (Laggner et al., 2000). This phorbol ester is a standard activator of the respiratory burst, and the authors showed that the respiratory burst was indeed the cause of the stimulation of DHA uptake in this animal system (Laggner et al., 2000, 2006). In the present study special interest was therefore shown in studying the relationship between Cd-induced ROS production and DHA uptake activity in plant cell cultures.

Studying the response of plant cell cultures to Cd has often resulted in the use of very high (millimolar range) concentrations of Cd, leading to complete cell death within 2–3 h of treatment (Piqueras et al., 1999; Olmos et al., 2003; Kuthanová et al., 2004; Garnier et al., 2006). The aim of the present study, however, was to analyse the response to Cd concentrations that did not lead to immediate cell death and to try to shed light on the earlier cellular responses that eventually lead to adaptive mechanisms. In this respect, it was shown that the response of cells to 1 mM or 50 µM CdCl$_2$ is completely different (Fojtová and Kovařík, 2000; Kuthanová et al., 2004). BY-2 cells died quickly after exposure to 1 mM without going through a process of PCD, whereas cell viability remained high for a couple of days in the presence of 50–100 µM Cd. However, when cell death was induced by 50 µM CdCl$_2$, this coincided with changes in polyamine content, fragmentation of nuclei, and incidence of TUNEL-positive nuclei. These are all typical features of PCD. More interestingly, Kuthanová et al. (2004) showed that the BY-2 cell population did not respond homogenously to 50 µM CdCl$_2$, with part of the cells being able to withstand and probably induce adaptive mechanisms. It was therefore chosen in the present study to work within the Cd concentration range that induced PCD in tobacco cell cultures. It is shown here that a 75 µM dose of Cd induced a rapid production of H$_2$O$_2$ at the plasma membrane. The overall redox status of the plant cells stayed high during the Cd treatment. However, GSH levels dropped dramatically, while phytochelatins were produced. It is further shown that although the overall ASC concentrations do not change, the uptake of DHA from the external medium drops. This response seems to be dependent on the uptake of Cd by the cells as this is prevented in La-treated cells. On the other hand, it is insensitive to diphenylene iodonium (DPI) treatment, indicating its independence of H$_2$O$_2$ production. Ideas on the physiological role of the changed DHA transport activity will be discussed.
Materials and methods

Plant material

Cell cultures of Arabidopsis thaliana were obtained from Dr F Van Breusegem (VIB, University of Ghent) and propagated in Gamborg’s B5 growth medium (3.2 g l\(^{-1}\)) at pH 5.7 (KOH), enriched with 30 g l\(^{-1}\) sucrose, 0.1 mg l\(^{-1}\) kinetin (stock in 1 M of NaOH), and 0.2 mg l\(^{-1}\) naphthaleneacetic acid (stock in 1 M of NaOH). Cell cultures were grown in sterile conditions in 250 ml Erlenmeyer flasks on a rotary shaker (New Brunswick Sci. Co.) at 22 °C at 100 rpm with a photoperiod of 16 h light and 8 h dark (50 \(\mu\)E m\(^{-2}\) s\(^{-1}\)). Every 7 d, cultures were subcultured by transferring 5 ml of culture to 50 ml of fresh medium. All experiments were performed with 3–4-d-old, exponentially growing cell cultures. Cells were harvested by filtering the cell suspensions over a Büchner filter with a cellulose filter (grade 1) and washed once with 5 mM MES-KOH buffer, pH 6.7, 22 °C) buffer. Harvested cells were resuspended at a concentration of 0.5 g fresh weight (FW) in 10 ml of the 5 mM MES-KOH buffer (pH 6.7, 22 °C).

Uploading of the cell cultures with ASC or GSH was performed as described by Horemans et al. (2003). Cells were incubated for 4 h in the loading medium, and immediately after this period used for further experiments.

H\(_2\)O\(_2\) analysis

The production of H\(_2\)O\(_2\) was measured spectrophotometrically as described in Raeymaekers et al. (2003), using 3,5-dichloro-2-hydroxybenzenesulphonic acid (DCHBS) and 4-aminointipyrine (AAP). Briefly, in the presence of H\(_2\)O\(_2\), DCHBS is oxidized by constitutive cell wall peroxidases to its quinone form, which specifically reacts with AAP to generate a molecular complex absorbing at 510 nm. The H\(_2\)O\(_2\) concentration was estimated by a calibration curve of H\(_2\)O\(_2\) standards.

Plant cell viability

In every experiment, the viability of the cell cultures was assayed by addition of Evans Blue to a final concentration of 0.05% (w/v). As Evans Blue can only permeate ruptured plasma membranes, it specifically invades dead cells. At 15 min after addition of Evans Blue, the percentage of living cells was determined by scoring at least 500 cells.

Quantification of cadmium uptake in cells

Cd concentrations were measured as described in Blust et al. (1988) with some modifications. Briefly, 4-d-old cells were collected as described above and resuspended in MES-KOH buffer at a concentration of 0.5 g FW in 10 ml of the 5 mM MES-KOH buffer (pH 6.7, 22 °C). CdCl\(_2\) and/or LaCl\(_3\) was added in the concentrations described in the figure legends. After 5 min or 3 h exposure to CdCl\(_2\), cells were harvested over a Büchner filter on a cellulose nitrate filter (0.45 μm, Sartorius) and washed three times with MES-KOH buffer. A 100 μg aliquot of fresh weight samples was collected into 14 ml polypropylene tubes using an acid-washed plastic spatula. For easy destruction, samples were freeze-dried during 48 h (Freeze Dry System, LABCONCO KS, USA). As quality control, three preparation blanks and three reference samples of olive leaves (BCR 062, EU Institute for Reference Materials and Measurements, Geel, Belgium) were included. For each sample, 250 μl of highly purified concentrated HNO\(_3\) was added, then placed in a fume hood to pre-digest during 24 h at room temperature. Further digestion was carried out by sequential heating in a microwave oven: three times 2 min at 100 W, followed by three times 2 min at 150 W and twice 2 min at 250 W. Then 20 μl of H\(_2\)O\(_2\) was added and the solutions were left for 30 min before giving them two additional 2 min microwave treatments at 350 W. After digestion, the solutions were diluted by adding 2 ml of MilliQ water, and Cd concentrations in the solutions were determined using an ICP-MS (Model 810, Varian Inc., Australia). Ytrrium was added as an internal standard to correct for the influence of sample matrix. The recovery of Cd in the reference samples was within the acceptable range of 10% of the certified values. As a background experiment, cells without CdCl\(_2\) added were also sampled and the Cd concentration was determined. No significant levels of Cd could be determined in these controls (data not shown).

HPLC analysis

ASC and GSH were determined by HPLC analysis. Cells were harvested over a Büchner filter and immediately frozen in liquid nitrogen. Fractions of 50–100 mg of frozen cells were ground in liquid nitrogen in a pre-cooled mortar. At this stage it is essential to keep samples frozen to prevent oxidation. A homogeneous powder was obtained, 400 μl of ice-cold 6% (w/v) meta-phosphoric acid was added. The samples were thawed on ice and the mixture was clarified by centrifugation at 16 000 g at 4 °C for 10 min. The resulting supernatant was kept frozen or on ice until HPLC analysis. Antioxidants were separated on a 100 mm × 4.6 mm Polaris C18-A reversed phase HPLC column (3 μm particle size; 30 °C; Varian, CA, USA) with an isocratic flow of 1 ml min\(^{-1}\) of the elution buffer (25 mM KPO\(_4\) buffer, pH 3.0). The components were quantified using a custom-made electrochemical detector with a glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The purity and identity of the peaks were confirmed using a diode array detector (SPD-M10AVP, Shimadzu, ‘s Hertogenbosch, The Netherlands) which was placed in line with the electrochemical detector. The concentration of oxidized DHA or GSH was measured indirectly as the difference between the total concentration of antioxidants in a dithiothreitol (DTT)-reduced fraction and the concentration in the sample prior to reduction. Reduction of the sample was achieved by incubation of an aliquot of the extract in 400 mM TRIS and 200 mM DTT for 15 min in the dark. The pH of this mixture was checked to be between 6 and 7. After 15 min, the pH was lowered again by 4-fold dilution in elution buffer prior to HPLC analysis.

DHA transport activity

Cells were harvested over a Büchner filter in order to remove Cd or other additions, and they were resuspended in Gamborg’s B5 medium at a concentration of 0.1 g ml\(^{-1}\). Uptake of DHA was measured according to Horemans et al. (1998) by adding 50 μM L-[\(^{14}\)C]ascorbic acid (Amersham, Ghent, Belgium) to 30 μl of freshly harvested cells (0.1 g ml\(^{-1}\)) in a final volume of 100 μl of B5 medium. As described in Horemans et al. (2003), the added L-[\(^{14}\)C]ASC is instantly oxidized to \([\(^{14}\)C]DHA in the presence of cells. Additional controls for this oxidation using ferricyanide together with \([\(^{14}\)C]ASC were also performed and showed no...
difference from the cells incubated with \([^{14}C]\)ASC alone (data not shown). This indicates that the different treatments did not alter the capacity of the cells to oxidize the \([^{14}C]\)ASC to \([^{14}C]DHA\).

After 20 min of incubation in the presence of radiolabelled DHA, cells were diluted 50-fold with ice-cold washing medium [10 mM of non-labelled ASC, Gamborg’s B-5 medium, pH 5.7 (KOHI)], collected on a Whatmann cellulose filter (grade 3M), and rinsed by further addition of 15 ml of washing medium. The filters were dissolved in scintillation cocktail (Filter Count: Packard, Brussels, Belgium). For background experiments, samples were washed immediately after addition of the radioactively labelled molecules. In a typical DHA uptake experiment, three replicates were made for each condition tested together with two background experiments, and an average was determined from at least three independent experiments.

**Real-time PCR**

Real-time PCR was performed to quantify transcription of \(RBOH\) (respiratory burst oxidase homologues) genes. Approximately 100 mg of 4-d-old cells, either control cells or treated with 75 \(\mu M\) CdCl\(_2\), were harvested over a Büchner filter and frozen in liquid nitrogen. RNA was isolated by the Concert Plant RNA Reagent for small-scale RNA harvest at different time points after Cd exposure as described in Sneller et al. (2000). Briefly cells were harvested at different time points after Cd exposure as described in Sneller et al. (2000). For background experiments, samples were washed immediately after addition of the RNA was further verified on an RNA gel, using standard procedures. From 2.5 \(\mu g\) to 5 \(\mu g\) RNA was loaded on the gels, showing a sharp distinction at the small side of both the 18S and 28S rRNA bands. Starting from equal amounts of RNA (1 \(\mu g\)), first-strand cDNA synthesis was primed with an oligo(dT)\(_{16}\) primer according to the manufacturer’s instructions using Taqman Reverse Transcription Reagents (Applied Biosystems). Quantitative PCR was performed with the ABI Prism 7000 (Applied Biosystems), Taqman chemistry. Primers and probes were designed and optimized by Assa-on-demand (Applied Biosystems), and the transcript level of the following genes was determined (Atg number and ABI reference number are given in parentheses): \(RBOHC\) (Atg5\(_{51060}\), Atg2319692\(_{m1}\)), \(RBOHD\) (Atg5\(_{47910}\), Atg2317797\(_{g1}\)), and \(RBOHF\) (Atg6\(_{4060}\), Atg2218138\(_{m1}\)). As a reference the \(UBQ10\) gene (Atg4\(_{05320}\), Atg2353586\(_{g1}\)) was used. PCR amplifications were performed in a total volume of 25 \(\mu l\), containing 5 \(\mu l\) of cDNA sample, 12.5 \(\mu l\) of Taqman Universal Master Mix (Applied Biosystems), 1.25 \(\mu l\) of assay mix (primers and probe), and 6.25 \(\mu l\) of RNase-free H\(_2\)O. All samples were tested in duplicate for the housekeeping gene following the 2\(^-\Delta\Delta Ct\) method (Livak and Schmittgen, 2001). It was evaluated that the level of expression of the housekeeping gene did not change under the experimental conditions used.

**Phytochelatin analysis**

Phytochelatin analysis was performed by Dr Schat, Laboratory of Plant Ecology and Physiology, Free University, Amsterdam, The Netherlands as described in Sneller et al. (2000). Briefly cells were harvested at different time points after Cd exposure as described above. About 25 mg of freeze-dried plant material was ground using a mortar and pestle, and subsequently extracted with 1.98 ml of 6.3 mM diethylenetriaminepentaacetic acid with 0.1% (v/v) trifluoroacetic acid at 4 \(^°\)C supplemented with 20 ml of 10 mM N-acetyl cysteine as an internal standard. The suspension was centrifuged at 13 000 \(g\) for 10 min at 4 \(^°\)C and the supernatant filtered through a Costar Spin-X centrifuge tube with a 0.22 mm nylon filter. The thiols in the extract were derivatized with 10 ml of 25 mM monobromobimane, together with 450 ml of 200 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid buffer at pH 8.2 and 6.3 mM diethylenetriaminepentaacetic acid. After 30 min at 45 \(^°\)C, the derivatization was stopped by the addition of 300 ml of 1 M methanesulphonic acid. The phytochelatins were separated on two tandemly arranged Nova-Pak C18 columns (6 nm, 4 \(\mu m\), 3.9×150 mm, Waters, Milford, MA, USA) at 37 \(^°\)C, using a slightly concave gradient of 12–25% (v/v) methanol for 15 min and then a linear gradient from 25 to 50% (v/v) methanol from 15 to 40 min. Fluorescence was monitored using a Waters 474 fluorescence detector. HPLC peaks were identified based on a Silene vulgaris sample in which the phytochelatin composition was established previously through amino acid analysis of the purified peak fraction (Sneller et al., 2000).

Data are mean values with SE from three independent experiments with three replicates each (unless indicated otherwise). Significance levels were tested by a two-tailed Student’s \(t\) test with \(\alpha\) of 0.05.

**Results**

**Induction of H\(_2\)O\(_2\) production by different metals**

Cell suspension cultures established the opportunity to administer and study the direct effects of metals at the cellular level, a condition that is often not the case in experiments involving whole plants. On the other hand, the composition of growth media or buffers can influence the amount of free metal ions in a solution. Prior to exposure to CdCl\(_2\), the \(A.\) thaliana cell cultures were therefore resuspended in MES, a buffer known to exhibit very low metal-binding properties (Soares et al., 2003), thereby minimizing the scavenging effect of the experimental medium on the metal ion concentrations.

Induction of H\(_2\)O\(_2\) production in \(A.\) thaliana cell cultures was tested after exposing the cells to different concentrations ranging from 10 \(\mu M\) to 100 \(\mu M\) of CdCl\(_2\) added to 4-d-old cell suspensions, and H\(_2\)O\(_2\) production was measured as a function of time after treatment. In Fig. 1 it is shown that this results in a rapid and strong increase in H\(_2\)O\(_2\) levels that is clearly dependent on the administered
Cd concentration. Concentrations as low as 10 μM CdCl₂ resulted in significant H₂O₂ production by the cell cultures. Using alternative salts (CdSO₄), it was shown that it was the Cd²⁺ and not the Cl⁻ component that induces the H₂O₂ production (data not shown). To investigate whether the observed production of H₂O₂ was related to an increased cell death, the cell viability was controlled at the end of every experiment. The cell viability after 5 h treatment with 75 μM CdCl₂ was comparable with the viability of non-treated cells (survival of 94±5% of the cells). Higher concentrations of Cd, however, caused a dose-dependent decrease in the cell viability (data not shown). As indicated in the Introduction, the focus of the present paper is on metal levels that do not immediately induce cell death, and therefore a concentration of 75 μM CdCl₂ was chosen for further experiments.

Localization and mechanism of H₂O₂ production induced by CdCl₂

The DCHBS/AAP-based method used above to detect H₂O₂ in the cell cultures will only measure extracellular H₂O₂, thus the subcellular localization of the H₂O₂ production remained unclear. The in vivo H₂O₂ production of CdCl₂-treated cell cultures was therefore visualized by fluorescence microscopy. The histochemical method used here measures the fluorescence of rhodamine 123 formed after the specific reduction of dihydrorhodamine by H₂O₂ (Henderson and Chappell, 1993). Figure 3 shows that the accumulation of H₂O₂ detected within 15 min after exposure of the cell cultures to 75 μM CdCl₂ is clearly at the periphery of the cells. From these images, it is impossible to conclude whether it is really extracellular. However, 1 h after the start of the treatment, H₂O₂ was present all over the cells. However, based on the present experiments, it is not possible to determine the subcellular localization of the Cd-induced ROS production or whether more than one ROS production site is induced.

The possible involvement of an NADPH oxidase-like enzyme complex in the Cd-induced oxidative burst was examined by testing the effect of DPI, an inhibitor of the mammalian NADPH oxidase complex, on H₂O₂ production. Pre-treatment of cell cultures for 5 min with different concentrations ranging from 10 μM to 20 μM of DPI resulted in a concentration-dependent decrease in the ROS production (measured with the DCHBS/AAP method, Fig. 2). Concentrations of 20 μM DPI reduced...
the H$_2$O$_2$ production in Cd-treated cells to the background level of control non-treated cells. In Fig. 3 it is shown that DPI-pre-treated cells did not show any fluorescent staining of the H$_2$O$_2$ production for at least 1 h after Cd addition.

At the transcriptional level, a gene expression analysis of three members of the RBOH family (RBOHC, D, and F) was performed. Fifteen minutes after the addition of 75 $\mu$M CdCl$_2$ the mRNA level of RBOHF doubled as compared with untreated cells. This increment was temporary; 3 h after the stress induction the gene was almost 5-fold down-regulated (Fig. 4). No significant difference in activity of RBOHD or RBOHC due to the Cd stress was detected within this time frame.

**Effect of acute Cd stress on the ascorbate and glutathione pool of the cells**

The antioxidative response of cell cultures to Cd treatment was studied by measuring the total ASC and GSH pool. It was hypothesized that high levels of H$_2$O$_2$ induced by acute Cd exposure could affect the antioxidative balance of the cells. The detected total ASC and total GSH contents of the cell cultures are presented in Figs 5 and 6. Interestingly, although no significant changes were observed in the total ASC pool, a very strong decrease in the GSH content is observed as soon as 1 h after Cd treatment. Together with the decrease in concentration, the pool of GSH became more oxidized whereas the total redox state of ASC was not affected compared with control cells (85–90% reduced, data not shown). It was tested whether increasing the concentration of internal ASC could prevent the decrease in GSH concentration. An increased internal ASC concentration in plant cell cultures can be obtained by incubating cells in the presence of 1 mM DHA for 4 h prior to administering Cd (Horemans et al., 2003). As stated by Horemans and coworkers (2003), once taken up, DHA is immediately converted to ASC. Pre-loading of the cells resulted in a significant increase (49±17%; Fig. 5) of the internal ASC concentration, as was expected, but had no effect on total GSH content nor on the Cd-induced depletion of GSH (Fig. 6). Uploading of the cells in a similar way with GSH (1 mM, 4 h prior to the test) resulted in an increase of the GSH content by 13±7%, but also did not prevent the drop in GSH concentration induced by Cd (data not shown).

As has been shown before, a decrease in GSH under metal stress is often correlated with an increased production of phytochelatins (Cobbett, 2000). In the present
experiments phytochelatin 4 increased strongly, already 1 h after Cd treatment. Also phytochelatin 5 showed an increase in concentration although less prominent compared with phytochelatin 4 (Fig. 7). No changes were found for phytochelatins 2 and 3.

Effect of acute Cd stress on the DHA uptake at the plasma membrane

ASC is known as a first line defence against external H₂O₂ production (Noctor and Foyer, 1992). Furthermore, the uptake of DHA is hypothesized to be important in the regeneration of apoplastic ASC (Horemans et al., 2000). Due to the high external H₂O₂ accumulation after exposing plant cells to 75 μM CdCl₂, it was of interest to determine the cell’s antioxidant levels as well as the uptake of DHA by the stressed cells. DHA uptake was measured as described by Horemans et al. (2003). No significant changes in DHA transport were observed in control cell cultures during the course of the experiment. However, a significant decrease in DHA transport activity was observed in the Cd-treated cell cultures after 2 h (Fig. 8). After 4 h, DHA uptake activity had dropped to only 10% of control levels. In a control experiment, the effect of Cd treatment on another known transport system, namely glucose uptake, was measured and shown not to change within the measured time frame (data not shown).

Since in plasma membrane vesicles it was hypothesized that the DHA transporter could act as an exchange carrier (Horemans et al., 1998), it is conceivable that changes in internal ASC levels could trigger changes in DHA uptake activity. Although Cd did not induce a significant change in total ASC levels (Fig. 5), it was tested whether the effect of Cd on DHA uptake activity changed in cells with higher internal ASC concentrations. A higher internal ASC concentration was obtained, as described above and shown in Fig. 5. Under these conditions, DHA transport increased slightly but not significantly with respect to the non-loaded controls tested immediately after uploading (data not shown). After 3 h, however, no difference in DHA uptake activity was present in control cells compared with loaded cells (Fig. 9). Loading the cell suspensions with DHA could not prevent the drop in DHA transport after Cd treatment (Fig. 9). This indicates that the decrease in DHA uptake activity is not triggered by changes in the internal ASC concentration.

The uptake of Cd into plant cells is known to be facilitated by Ca channels (Perfus-Barbeoch et al., 2002). As such, many studies have shown that LaCl₃, an inhibitor
of Ca channels, prevents Cd-induced responses. Addition of 1 mM LaCl₃ to the cell cultures 5 min prior to Cd treatment effectively inhibits Cd uptake into the cells (Table 1). After 5 min in the presence of CdCl₂, cells treated with LaCl₃ only showed ~5% of the Cd taken up by the cells without LaCl₃ treatment (Table 1). This inhibition persisted as, after 3 h, Cd uptake was still inhibited by >90% (Table 1). Similarly to the results obtained by Garnier et al. (2006), treatment of the cells with 1 mM LaCl₃ also prevented the Cd-induced oxidative burst (data not shown). Here the effect of La on the interaction between Cd treatment and DHA uptake activity in cells was investigated. Pre-treatment of the cells with 1 mM LaCl₃ 5 min before exposing them to Cd prevented the Cd-induced drop in DHA uptake activity (Fig. 9). As indicated above, the Cd-induced ROS production is sensitive to the flavin inhibitor DPI. However, pre-treatment of the cell culture with 20 μM DPI did not prevent the decrease in DHA uptake activity induced by Cd (Fig. 9). Addition of LaCl₃ or DPI alone did not affect the DHA uptake capacity of control cells (data not shown).

**Discussion**

A relationship between oxidative stress, redox homeostasis, and heavy metal toxicity has been repeatedly reported in the literature for plants as well as plant cell cultures (for a review, see Schützendübel and Polle, 2002). A general conclusion drawn from the data reviewed by Schützendübel and Polle (2002) was that metals such as Cd, when not detoxified rapidly enough, affect redox homeostasis and through this may trigger reactions leading to growth inhibition, lipid peroxidation, lignification, and finally cell death. Specifically for Cd, it has been shown that, depending on the Cd concentration applied, the cytotoxicity it exerts on cell cultures varies (Fojtová and Kovařík, 2000; Kuthanova, 2004). As indicated in the Introduction, suspended plant cells treated with Cd concentrations of 50–100 μM were partially able to survive this treatment or go through a process of PCD (Fojtová and Kovařík, 2000; Kuthanova, 2004). In contrast, 1 mM and 5 mM CdCl₂ induced an almost immediate necrotic cell death that was accompanied by an oxidative burst (Piqueras et al., 1999; Olmos et al., 2003; Garnier et al., 2006). However, to date, it has not been shown whether short-term treatments with lower Cd concentrations also induce an oxidative burst in cell cultures comparable with that in cells challenged with higher concentrations. Here it is shown that exposure of A. thaliana cell cultures to 75 μM CdCl₂ results in rapid H₂O₂ production (within 5 min of treatment). Comparable with the results of Fojtová and Kovařík (2000) and Kuthanová (2004), the viability of the cells remained as high as control levels for at least 5 h after inducing Cd stress on the Arabidopsis cells.

This oxidative burst is initially localized at the periphery of the cells (Fig. 3) and can be completely inhibited by DPI. DPI is known as an inhibitor of the mammalian neutrophil NADPH oxidase complex. It is, however, a general inhibitor of proteins containing flavins, and therefore lacks selectivity for the NADPH oxidase complex (Raeymaekers et al., 2003). In order to correlate ROS production with NADPH oxidase-like enzyme activity, the expression of different RBOH genes was followed. Since their identification, the 10-member RBOH family has been implicated in biotic interactions, abiotic stress responses, and plant development (for reviews, see Davies et al., 2006; Sagi and Fluhr, 2006; Torres et al., 2006). In the present study it is shown for the first time that the Cd-triggered oxidative burst is accompanied by a transient increase in the expression of RBOHF (Fig. 4), whereas the expression of RBOHC and D did not change. RBOHF and RBOH D are known to be constitutively expressed throughout the plant and were identified as required for a full oxidative burst in response to pathogens (Torres et al., 2002). Changes in transcript levels of RBOHC, D, and F were detected in leaves and roots of 3-week-old Arabidopsis plants exposed to 10 μM CdSO₄ for 24 h (A Cuypers, personal communication). Taken together, these results indicate that an NADPH oxidase-like enzyme is involved in the oxidative burst induced by 75 μM Cd in plant cell cultures, similar to that observed in BY-2 cell cultures treated with high concentrations (Piqueras et al., 1999; Olmos et al., 2003; Garnier et al., 2006). Although during the first hour, ROS production is completely inhibited by DPI, it remains to be investigated whether different ROS-producing mechanisms come into play at later time points, as has been described for BY-2 cells challenged with millimolar concentrations of Cd (Garnier et al., 2006).

The concentration and redox state of the two most abundant antioxidants in plants (ASC and GSH) were determined in Cd-treated cells. Cd induced a depletion of the GSH pool within 2–3 h after the start of the treatment. Similar results have been found in whole plants (Schützendübel et al., 2001; Schützendübel and Polle, 2002; Hsu and Kao, 2005). Although GSH can act as a direct scavenger of ROS, the early decrease in its level is probably attributable to an increased consumption for

**Table 1. Effect of LaCl₃ (1 mM) on the uptake of Cd in 4-d-old cells exposed to 75 μM CdCl₂ for 5 min or 3 h**

<table>
<thead>
<tr>
<th>CdCl₂</th>
<th>+CdCl₂ +LaCl₃</th>
<th>5 min</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.3 ± 3.3</td>
<td>0.77 ± 0.11 (5.03%)</td>
<td>89.0 ± 7.1</td>
<td>8.3 ± 2.1 (9.3%)</td>
</tr>
</tbody>
</table>

The average of three independent experiments with SE is shown. The percentage of Cd taken up in cells in the presence of LaCl₃ compared with cells without LaCl₃ is given in parentheses.
phosphatidylcholine production (Zenk, 1996; Nocito et al., 2002). This correlates with Fig. 7, showing an increase in phosphatidylcholine 4 and 5 concentrations after Cd treatment. Although the redox status of GSH decreased, the redox status of ASC remained high during the experiments. Incubating cells in ASC or GSH before exposure to Cd further showed that the decrease in GSH cannot be prevented by higher internal ASC or GSH concentrations. This indicates that it is probably not an imbalance in the redox homeostasis that triggers this response (Fig. 6). Other studies have indicated that the depletion of GSH is transient and that GSH levels tend to increase in long-term experiments. For example, Pietrini et al. (2003) demonstrated a 37-fold increase in total leaf GSH levels in leaf discs floating on 40 μM Cd incubation medium for 3 d. Interestingly, it has been suggested that the drop in GSH level is a critical step in the Cd-induced ROS production (Schützendübel and Polle, 2002). In our hands, however, significant ROS production is measurable as soon as 5 min after Cd addition, and thus before the drop in GSH concentration.

In contrast to changes in GSH, no significant changes in ASC levels or ASC redox status (not shown) were present when Arabidopsis cells were treated with 75 μM Cd. This is remarkable given the fact that significant levels of H₂O₂ were produced. Care must be exercised as total ASC levels may hide more subtle changes within one cell compartment. For example, it is conceivable that as H₂O₂ is primarily produced in the apoplast, changes in apoplastic antioxidants could be present. In a first attempt to study changes at the level of the plasma membrane, the uptake of DHA in the Cd-treated Arabidopsis cells was investigated. The DHA transporter present in higher plant plasma membranes could act as an exchange carrier (Horemans et al., 1998), possibly contributing to the regeneration of external ascorbate. In animal cells it was shown that uptake of DHA increased when HL-60 cells were treated with an activator of the respiratory burst (Laggner and Goldenberg, 2000). This increase was triggered by the production of superoxide by the NADPH oxidase, as evidenced by inhibition by DPI (Laggner et al., 2000) and by mutual sensitivity (both NADPH oxidase and the change in DHA uptake) to zinc (Laggner et al., 2006). In the HL-60 cells, the increased DHA uptake was due not only to a higher DHA concentration but also to a simultaneous recruitment of additional transporter molecules to the cell surface (Laggner and Goldenberg, 2000).

In our Arabidopsis cell cultures, Cd treatment resulted, in contrast, in a significant decrease in DHA uptake activity. This decrease in DHA uptake activity was not present in cells pre-treated with LaCl₃ (Fig. 9). LaCl₃ is a Ca channel blocker and also prevents the uptake of Cd in our cells (Table 1) and the induction of an oxidative burst by Cd (data not shown; Garnier et al., 2006). On the other hand, DPI, used in similar conditions that inhibited ROS production, did not prevent the Cd-induced drop in DHA uptake activity. These results indicate that in the Arabidopsis cells uptake of Cd is a prerequisite to induce a change in DHA uptake as well as an oxidative burst, but that these two responses are independent of each other. The lower DHA uptake activity observed was not attributable to alterations in internal ASC levels as these did not change significantly upon Cd treatment (Fig. 9). Additionally, pre-loading the cells with GSH could also not prevent the drop in DHA uptake activity induced in Cd-treated cells (data not shown). GSH has been shown to be involved in the reduction of DHA to ASC (Noctor and Foyer, 1998). However, results on BSO-treated tobacco BY-2 cells showed that the uptake of DHA over the membrane occurred independently of the cytosolic glutathione concentration (Potters et al., 2004). Here, it still remains to be established whether the change in DHA uptake activity is a direct effect of the Cd exposure or whether it is triggered by the decrease in glutathione levels induced by Cd treatment.

In animal cells, DHA is transported by facilitative glucose transporters (GLUT1, 2, and 4; Vera et al., 1993). In plant cell cultures, it has been shown that the activity of plant glucose transporters changes in stress conditions. Azevedo and co-workers (2006) demonstrated enhanced glucose uptake activity in Pinus pinaster suspended cells infected with the non-host-specific pathogen Botrytis cinerea. This enhanced uptake was dependent on NADPH oxidase activity and on Cu²⁺ influx (Azevedo et al., 2006). In contrast, treating tobacco cells with cryptogein, an elicitor of the hypersensitive response in these cells, blocked glucose uptake (Bourque et al., 2002). Very similar to our results, the drop in glucose uptake in these tobacco cells was sensitive to La³⁺ but not to DPI, indicating the involvement of Ca channels but not of the oxidative burst (Bourque et al., 2002). For plant cells, a relationship between GLUTs and DHA transport has not been shown so far but will be an interesting subject for future experiments.

In conclusion, in this study Arabidopsis cell cultures were challenged with Cd concentrations known not to induce immediate cell death. These stressed cells showed an immediate oxidative burst dependent on NADPH oxidase activity, phosphatidylcholine production, and, within 4 h, inhibition of the uptake of DHA from the medium. Together with the fact that the plant DHA transport activity varies depending on the cell cycle (Horemans et al., 2003), these results indicate that this transporter is a highly regulated protein in plant plasma membranes possibly involved in the interrelationship between ASC redox state and the response of plant cells to the environment.

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References


