Sulphate assimilation under Cd\textsuperscript{2+} stress in *Physcomitrella patens* – combined transcript, enzyme and metabolite profiling

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**ABSTRACT**

Cd\textsuperscript{2+} causes disturbance of metabolic pathways through severe damage on several levels. Here we present a comprehensive study of Cd\textsuperscript{2+}-mediated effects on transcript, enzyme and metabolite levels in a plant without phytochelatin (PC). The moss *Physcomitrella patens* (Hedw.) B.S.G. was stressed with up to 10 \(\mu\)M Cd\textsuperscript{2+} to investigate the regulation of gene transcription and activities of enzymes involved in the assimilatory sulphate reduction pathway and in glutathione biosynthesis. *Real-time* PCR, specific enzyme assays as well as thiol peptide profiling techniques were applied. Upon supplementation of 10 \(\mu\)M Cd\textsuperscript{2+}, the moss showed a more than fourfold increase in expression of genes encoding ATP sulphurylase (ATPS), adenosylphosphosulphate reductase, phosphoradenosylphosphorosulphate reductase, sulphite reductase (SiR) and \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-ECS). Likewise, elevated enzyme activities of \(\gamma\)-ECS and glutathione synthetase were observed. Contrarily, activity of O-acetylsertine (thiol) lyase (OAS-TL), responsible for biosynthesis of cysteine, was diminished. At the metabolite level, nearly doubling of intracellular cysteine and glutathione content was noted, while the moss did not produce any detectable amounts of PCs. These results suggest a Cd\textsuperscript{2+}-induced activation of the assimilatory sulphate reduction pathway as well as of glutathione biosynthesis on different levels of regulation.

Key-words: glutathione.

**INTRODUCTION**

To deal with a great variety of heavy metal ions as natural components of the biosphere, plants had to evolve sophisticated defence strategies. While some of these metals (e.g. Zn, Mn, Cu, Fe) are essential for metabolism (e.g. as cofactors or activators of enzymes), others (e.g. Cd, Pb, Hg) are toxic even at very low concentrations. For instance, Cd\textsuperscript{2+} can cause disturbances of metabolic pathways such as photosynthesis, C, N and S metabolism (Sanità Di Toppi & Gabbrielli 1999; Astolfi, Zuchi & Passera 2004b; Prasad 2004a; Mendoza-Cózat et al. 2005), as well as changes in the redox state (Tausz, Sircelj & Grill 2004). In general, heavy metals can be detoxified by avoidance or tolerance mechanisms. Plants avoid heavy metal uptake by root exudates to complex metals or by immobilization on cell walls, whereas plant tolerance to toxic and excess essential metals is based on a strict regulation of cellular metal homeostasis (Hall 2002; Mallick & Rai 2002; Callahan et al. 2006). Detoxification of Cd\textsuperscript{2+} and other toxic soft Lewis acid metal ions by thiol peptides provides a distinct cytoplasmic mechanism (Cobbett & Goldsbrough 2002; Tausz 2003; Prasad 2004b; Mendoza-Cózat et al. 2005). Herein, glutathione (GSH)-derived metal-complexing phytochelatins (PCs) [general structure: (\(\gamma\)-GluCys)\(_2\)Gly] play an important role in plants and fungi (Cobbett & Goldsbrough 2002; Hall 2002; Clemens & Simm 2003; Clemens 2005; Jaeckel et al. 2005; Mendoza-Cózat et al. 2005). Heavy metal detoxification is finally achieved by active transport of metal PC complexes into plant or fungal vacuoles (Ortiz et al. 1995; Salt & Rauser 1995; Clemens & Simm 2003). Recent investigations of Cd\textsuperscript{2+}-stressed bryophytes showed no PC biosynthesis, but a remarkable increase in the GSH pool (Bruns et al. 2001; Bleuel et al. 2005). In the aquatic moss *Fontinalis antipyretica*, there is evidence for cytosolic localization of sulphhydril (SH)-group chelated Cd\textsuperscript{2+} (Bruns et al. 2001). Most likely, GSH itself is able to bind Cd\textsuperscript{2+} in vivo and thus represents a substantial Cd\textsuperscript{2+} detoxification agent in mosses. For instance, the ectomycorrhizal fungus *Paxillus involutus* reacts upon Cd\textsuperscript{2+} stress by (1) a remarkable increase of GSH and its precursor, \(\gamma\)-glutamylcysteine (\(\gamma\)-EC), accompanied by a complete lack of PCs (Courbot et al. 2004); and (2) a high Cd\textsuperscript{2+} content in vacuoles (Blau dez, Botton & Chalot 2000). The ATP-binding cassette (ABC)-type transporter, yeast Cd factor (YCF1), catalyses the translocation of bis(glutathionato)Cd into vacuoles as shown for Saccharomyces cerevisiae (Li et al. 1997). Activity of YCF1-like proteins can be observed in higher plants as well, and the importance of YCF1/bis(glutathionato)Cd in phytoremediation was emphasized (Song et al. 2003; Tong, Kneer & Zhu 2004). Overexpression of YCF1 in *Saccharomyces* also rendered cells more resistant to Cd\textsuperscript{2+} than wild-type cells, and cells accumulated more Cd\textsuperscript{2+}. *Arabidopsis thaliana*
overexpressing YCF1-like proteins results in enhanced Cd\(^{2+}\) tolerance (Song et al. 2003).

The moss *Physcomitrella patens* is an emerging model organism for studying plant metabolism. This ancient bryophyte responds to Cd\(^{2+}\) stress differently than other model plants such as *A. thaliana*. This is mainly caused by the lack of PCs in *P. patens*. Therefore, different mechanisms of metal detoxification must exist in this organism.

In all plants, a dynamic pool of GSH is participating in multiple metabolic processes. GSH was previously found to be involved in heavy metal detoxification (Grill, Tausz & Penninckx 2004). As shown by *in silico* expressed sequence tag (EST) to peptide mass fingerprint comparison, a cysteine-rich protein might be involved in Cd stress response (Cho et al. 2006).

However, GSH is synthesized from inorganic sulphate via reductive sulphate assimilation (Fig. 1). This reduction responds to both changes in sulphur supply and environmental conditions that alter the reduced sulphur demand (Leustek et al. 2000; Leustek 2002; Rausch & Wachter 2005). When sulphate enters the cell by sulphate transporters (ST), ATP sulphurylase (ATPS) catalyses its adenylylation producing adenosine 5′-phosphosulphate (APS). APS reduction is consecutively carried out by two enzymes. Firstly, APS reductase (APR) transfers two electrons to APS, resulting in sulphite. Secondly, sulphite is reduced by six electrons to sulphide through sulphite reductase (SiR). Sulphide is incorporated by O-acetylserine (thiol) lyase (OAS-TL) into the serine acetyltransferase (SAT) product, OAS, to form cysteine, thereby connecting N and S metabolism (Kopriva & Rennenberg 2004). While APS is attributed to GSH in fungal metal tolerance (Pocsi, Prade & Penninckx 2004), we used liquid medium. As shown by *in silico* expressed sequence tag (EST) to peptide mass fingerprint comparison, a cysteine-rich protein might be involved in Cd stress response (Cho et al. 2006).

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Vitality and metal accumulation

Cd²⁺ influence on *P. patens* vitality was assayed by determining chlorophyll *a* and *b* content as well as effective quantum yield of photosystem II (PSII) [pulse amplitude modulation (PAM), ratio of variable to maximum fluorescence (*Fv/Fm*); Schreiber, Schliwa & Bilger 1989], which is measured as relative electron transport rate and fluorescence-quenching coefficients using a Mini PAM (Walz, Effeltrich, Germany). Thus, 0, 5 and 10 µM Cd²⁺ (concentration in the medium) were chosen as control, tolerable stress and stress with loss of vitality. To examine bioeffect of metal concentrations, intracellular Cd²⁺ contents were determined after washing the plant material with 20 mM NiCl₂ to remove adsorbed Cd²⁺ according to Bruns *et al.* (Bruns *et al.* 2001).

Primer design

Specific primers for genes coding for enzymes involved in *P. patens* sulphate reduction pathway and GSH synthesis were designed with Fast PCR software version 3.6.28 (Ruslan Kalendar, University of Helsinki, Finland). Firstly, Fast PCR provided several candidates for the primer-based EST sequences listed in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov, see Table 1 for GenBank accession identifications) from *P. patens*. It was determined that apr and papr are single-copy genes in *P. patens* (Koprivova *et al.* 2002). In *A. thaliana* and *Brassica juncea*, gsh1 and gsh2 are single-copy genes, too (Wachter *et al.* 2005). Because of this and its lower grade of gene redundancy in the moss compared with higher plants, it is very likely that at least, gsh1 and gsh2 are also single-copy genes in *P. patens*. For OAS-TL and SAT, there might be a second gene encoded by the genome of *P. patens*. However, no transcript was detectable for the second OAS-TL homolog in *P. patens* (unpublished results). Secondly, oligomer sequences were checked with an oligonucleotide properties calculator (http://www.basic.north-western.edu/biotools/oligocalc.html) to avoid hairpins and primer dimers. Resulting pre-optimal qPCR primers were optimized regarding annealing temperature to amplify one specific product for each mRNA in a single PCR run. Sequences of the primers used in this study are shown in Table 1.

RNA extraction

To isolate total RNA from *P. patens* cultures, RNase Plant Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s protocol. Final RNA concentration was determined with high scrutiny (*n* = 9). An amount of 0.75 µg total RNA was treated with DNase I (MBI Fermentas, Burlington, Canada) to avoid products from genomic DNA amplification in subsequent reactions.

Reverse transcription and quantitative PCR

DNA-free RNA (0.5 µg) was reverse transcribed using SuperScript II (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol.

Initial template amounts were quantified using real-time PCR (one-step QuantiTect SYBR Green PCR kit, Qiagen). Quantification was optimized following Bustin’s (2002) suggestions. In short, reaction mixture contained 12.5 ng cDNA as template, 10 µL 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen GmbH, Hilden, Germany), 0.5 µM forward primer, 0.5 µM reverse primer and nucleaease-free water in a final volume of 20 µL. Quantitative PCR was carried out using an iCycler (Bio-Rad, Munich, Germany). Specific conditions are available on request. To specifically check PCR products, the program included melting curves as proposed by Gachon, Mingam & Charrier (2004). Experiments were performed from cDNAs prepared from three independent batches of moss cultures.

Data analysis parameters

Threshold cycles were calculated by iCycler software (Bio-Rad). *Ct* values were used to quantify mRNA. The ∆∆*Ct*

<table>
<thead>
<tr>
<th>EST (NCBI)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>Rubisco</td>
<td>5′-AAGTGGCCCATGTTCGGGTG-3′</td>
<td>5′-ACCTGGTTCTTGTGCTGAGAG-3′</td>
</tr>
<tr>
<td>ATPS</td>
<td>5′-TTCTGGAACCTAGGCTGC-3′</td>
<td>5′-TCGAGTTTTTTGCACCTCCTGG-3′</td>
</tr>
<tr>
<td>APR</td>
<td>5′-ACGGTTGGTGAGTTTTCGAC-3′</td>
<td>5′-TGGTTGTATGCGGCTTGGTGTC-3′</td>
</tr>
<tr>
<td>PAPSR</td>
<td>5′-TCAACTGCGAGAATTTGCC-3′</td>
<td>5′-TCGAAACACACAGCATGCC-3′</td>
</tr>
<tr>
<td>SIR</td>
<td>5′-GGGTATTTCTCAAGGAG-3′</td>
<td>5′-TCATACGGGACATTCTGCGA-3′</td>
</tr>
<tr>
<td>SAT</td>
<td>5′-GTACAGCTGCTGGGAATCTC-3′</td>
<td>5′-ACCAGCCTTATGTGATTTGAC-3′</td>
</tr>
<tr>
<td>OAS-TL</td>
<td>5′-GGACGAGGTGGTGGATGAC-3′</td>
<td>5′-AAATTTGGCGGTGACATCTCCT-3′</td>
</tr>
<tr>
<td>γ-ECS</td>
<td>5′-TCTTTTGAGAAAAACACCAGCTC-3′</td>
<td>5′-CATGCTCCCAAACGAGTCCC-3′</td>
</tr>
<tr>
<td>GS</td>
<td>5′-ACCACCTATCATGCTCAG-3′</td>
<td>5′-TGAATTGGGCAATCTTGCCA-3′</td>
</tr>
</tbody>
</table>

*National Center for Biotechnology Information (NCBI) GenBank access identification. Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (housekeeping gene), enzymes of sulphate assimilation pathway and glutathione (GSH) biosynthesis used for quantitative reverse transcriptase (RT)-PCR.
method with the ribulose 1,5-bisphosphate carboxylase/oxgenase (Rubisco) gene as internal reference was applied as described by Livak & Schmittgen (2001).

High-performance liquid chromatography (HPLC) of thiols

Analysis of thiol compounds was carried out by HPLC using monobromobimane derivatization (Newton & Fahey 1995). Approximately 50 mg plant material was ground in liquid N, placed into 1 mL of 0.1 N HCl and centrifuged for 10 min at 20 000 g and 4 °C. Supernatant (120 µL) was added to 180 µL 0.2 M 2-(cyclohexyl-amino)ethanesulfonate (CHES) buffer, pH 9.3 and 30 µL 5 mM dithiothreitol (DTT). The mixture was incubated on ice for 1 h. Then, 10 µL monobromobimane (30 mM in MeOH) was added for thiol derivatization and incubated 15 min in the dark at room temperature. The reaction was stopped with 5% acetic acid (v/v).

HPLC analysis was carried out on a Lichrospher 60 RP Select B column (4 × 250 mm, 5 µm; Merck, Darmstadt, Germany) using a Merck Hitachi LaChrom system containing D-7000 interface, L-7100 pump, L-7200 autosampler and D-7480 fluorescence detector (λ exc = 420 nm, λ em = 520 nm). Mobile phase A consisted of 2% MeOH (v/v) in H 2O with 10 mM MgCl2, 1 mM EDTA followed by centrifugation at 10 000 g for 15 min.

The reaction mixture, in a final volume of 250 µL containing 100 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0), 10 mM MgCl2, 1 mM EDTA followed by centrifugation at 10 000 g and 4 °C for 15 min. The γECS reaction was carried out at 30 °C in a final volume of 250 µL containing 100 mM (Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) (pH 8.0), 50 mM MgCl2, 20 mM glutamate, 1 mM cysteine, 5 mM ATP, 5 mM phosphoenolpyruvate (PEP), 5 mM DTT and 10 U mL−1 pyruvate kinase. The reaction was started by adding 75 µL extract. The reaction times were 5 min (t0) and 65 min. The thiols were labelled with monobromobimane with 1 µU activity corresponding to 1 nmol γ-EC × min−1 at 30 °C. Solutions with varying γ-EC amounts were used for quantification.

To measure GS activity, the reaction was initiated by adding 25 µL extract to obtain a final volume of 250 µL containing 100 mM Tris/HCl (pH 8.4), 50 mM KCl, 20 mM MgCl2, 1 mM γ-EC, 2 mM Gly, 5 mM ATP, 5 mM PEP, 5 mM dithioerythritol (DTE) and 10 U mL−1 pyruvate kinase (30 °C). The reaction times were 5 min (t0) and 35 min. GSH was also determined via monobromobimane derivatization with 1 µU activity corresponding to 1 nmol GSH × min−1 at 30 °C.

Statistical analysis

Data were analysed with InStat (GraphPad, version 3.05). All values reported in this paper are averages of at least three independent experiments. SDs were included in the tables. Data of control and Cd2+ stressed experiments were compared and tested for significance by one-way analysis of variance (ANOVA).

RESULTS

Vitality of P. patens upon Cd2+ exposure

To estimate the ability of P. patens to resist Cd2+ stress, chlorophyll content and PSII activity were measured. Cd2+ ions caused a rapid decrease of chlorophyll: up to 20 (29)% of chlorophyll a and 22 (28)% of chlorophyll b were degraded within 3 d (Table 2) in the presence of 5 (10) µM Cd2+ compared to control plants. Longer incubation times were accompanied by even higher and more significant chlorophyll degradation rates. After 3 d incubation with 5 µM Cd2+, PSII activity was slightly reduced to 98% of that of the control plants, whereas 10 µM Cd2+ was accompanied by a loss of viability (93% activity, data not shown). P. patens is more sensitive to Cd than A. thaliana. Upon application of 15 µM Cd2+ for 3 d in liquid culture, most of the cells died and a brown colouring was noticed (data not shown).

In contrast to other mosses (e.g. F. antipyretica), P. patens suffered a rapid accumulation of Cd2+: the intracellular values for 5 (10) µM Cd2+ were 0.7 (1.3) µmol g−1 FW after 1 d; 0.8 (1.5) µmol g−1 FW after 3 d and finally 0.8 (1.4) µmol g−1 FW after 5 d of culture, respectively.

Gene transcription of assimilatory sulphate reduction pathway and GSH biosynthesis are induced upon Cd2+ exposure

We investigated transcript concentrations of cysteine and GSH biosynthesis genes to assess how Cd2+ affects gene
transcription in *P. patens*. Treatment with Cd\(^{2+}\) influenced transcription of genes coding for enzymes involved in sulphate reduction and GSH biosynthesis in time- and concentration-dependent manners (Fig. 2).

Exposure to Cd\(^{2+}\) for 24 h was accompanied by only moderate changes. However, especially transcripts for sulphate assimilation enzymes (e.g. ATPS, APR and SiR) showed a remarkable induction after 72 h. Finally, 5 d after Cd\(^{2+}\) addition, the transcription rates of genes involved in sulphate assimilation from 5 \(\mu\)M Cd\(^{2+}\)-treated plants were almost as high as from 10 \(\mu\)M Cd\(^{2+}\) treated plants (APR, phosphoadenosine 5’-phosphosulphate reductase (PAPR), SiR) or even slightly higher (SAT, OAS-TL). Compared with the control, 3 d exposition with 5 \(\mu\)M Cd\(^{2+}\) (tolerable stress) is accompanied by increased ATPS, APR, PAPR and SiR mRNAs at ratios of 1.4-, 2.4-, 4.0- and 4.0-fold, respectively. At 10 \(\mu\)M Cd\(^{2+}\) (stress with loss of vitality), SiR transcription was almost 10-fold increased after the same time period, whereas ATPS, APR and PAPR showed a more than fourfold transcript increase compared to control plants.

Cysteine is the end product of the sulphate assimilation pathway. In addition to its primary role in protein biosynthesis, cysteine is a precursor of GSH. Thus, elevated expression of genes coding for enzymes involved in this pathway can be anticipated in bryophytes. Indeed, γ-ECS and GS are up-regulated after Cd\(^{2+}\) stress (Fig. 2). The expression of both transcripts was uniformly low after 24 h. Only 10 \(\mu\)M Cd\(^{2+}\)-treated cultures showed significant threefold increase in the amount of γ-ECS mRNA. In contrast, 3 d of Cd\(^{2+}\) stress was accompanied by remarkable 5.7- (7.9-) fold stimulation of γ-ECS transcription and 2.2- (3.2-) fold induction of GS transcription at 5 (10) \(\mu\)M Cd\(^{2+}\), respectively. The results of 5 d treatment resembled those tendencies for sulphate assimilation (i.e. slight differences between 5 and 10 \(\mu\)M Cd\(^{2+}\)). However, their expression was nevertheless elevated.

**Thiol pools are increased after Cd\(^{2+}\) stress**

To analyse whether the induction of gene expression is reflected at the metabolite level, thiol profiling was carried out using reversed phase (RP)-HPLC of monobromobimane thiol derivatives. It was demonstrated that the contents of thiol metabolites increased time dependently (Table 3).

After 24 h, Cd\(^{2+}\)-stressed cultures contained higher cysteine pools than control plants. In contrast, 1 d heavy metal exposure was only reflected by slight changes of γ-EC and GSH, whereas 72 h after Cd\(^{2+}\) addition, these metabolites were clearly increased. Most notably, 10 \(\mu\)M Cd\(^{2+}\) was accompanied by significantly enhanced levels of cysteine, γ-EC (5 d) and GSH (3 and 5 d).

**Activities of cysteine and GSH-synthesizing enzymes are differently affected by Cd\(^{2+}\)**

To examine whether gene expression is reflected by higher enzyme activities, assays of key enzymes were adapted for *P. patens*. When cell extracts were analysed, a time-dependent differentiation of stressed and control plants could be observed (Table 4).

After 72 h of Cd\(^{2+}\) exposure, the activity of the cysteine-generating enzyme, OAS-TL, was significantly decreased to 79% (5 \(\mu\)M Cd\(^{2+}\)) or 77% (10 \(\mu\)M Cd\(^{2+}\)) compared to control plants, an effect emphasized after 5 d Cd\(^{2+}\) exposure.

Contrarily to this result, activities of γ-ECS and GS in *P. patens* were barely increased after 24 h (Table 4). Higher activities were observed only after longer incubation times. Compared to the control, 3 d of 5 (10) \(\mu\)M Cd\(^{2+}\) is accompanied by 1.8- (1.7-) fold γ-ECS activity, whereas 5 d 10 \(\mu\)M Cd\(^{2+}\) led to significant, 3.4-fold increased activity.

In GS, the Cd\(^{2+}\)-stressed cultures showed higher enzyme activities after 3 (5) days, too. Either of the Cd\(^{2+}\) concentrations was accompanied by significant 1.3- (1.4-) fold

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**Table 2.** Effect of different Cd\(^{2+}\) concentrations on chlorophyll *a* and *b* content [\(\mu\)g g\(^{-1}\) fresh weight (FW)] and chlorophyll ratio (*a/b*) in *Physcomitrella patens* after 1, 3 and 5 d of incubation

<table>
<thead>
<tr>
<th>Time after Cd(^{2+}) application (day)</th>
<th>Control</th>
<th>+5 (\mu)M Cd(^{2+})</th>
<th>+10 (\mu)M Cd(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll <em>a</em></td>
<td>1.91 ± 0.23</td>
<td>1.99 ± 0.19</td>
<td>1.77 ± 0.32</td>
</tr>
<tr>
<td>Chlorophyll <em>b</em></td>
<td>1.00 ± 0.10</td>
<td>0.99 ± 0.11</td>
<td>0.92 ± 0.15</td>
</tr>
<tr>
<td>Chlorophyll (<em>a/b</em>)</td>
<td>1.90</td>
<td>2.01</td>
<td>1.93</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll <em>a</em></td>
<td>2.04 ± 0.21</td>
<td>1.63 ± 0.18</td>
<td>1.46 ± 0.22*</td>
</tr>
<tr>
<td>Chlorophyll <em>b</em></td>
<td>1.04 ± 0.10</td>
<td>0.81 ± 0.09</td>
<td>0.75 ± 0.09*</td>
</tr>
<tr>
<td>Chlorophyll (<em>a/b</em>)</td>
<td>1.96</td>
<td>2.02</td>
<td>1.95</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll <em>a</em></td>
<td>2.13 ± 0.08</td>
<td>1.48 ± 0.17**</td>
<td>1.16 ± 0.20***</td>
</tr>
<tr>
<td>Chlorophyll <em>b</em></td>
<td>1.06 ± 0.02</td>
<td>0.74 ± 0.09**</td>
<td>0.62 ± 0.07***</td>
</tr>
<tr>
<td>Chlorophyll (<em>a/b</em>)</td>
<td>2.00</td>
<td>2.00</td>
<td>1.85</td>
</tr>
</tbody>
</table>

\(n = 3\).

*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\).*
increased enzymatic activity after 72 h. Maximum GS activity was reached at 5 d 10 µM Cd²⁺ with a 1.6-fold significant higher activity compared to control plants.

**DISCUSSION**

The aim of this study was to obtain information about Cd²⁺-modulated transcription of genes encoding enzymes of sulphate assimilation and GSH biosynthesis. To assess the responses on cellular metabolism, thiol contents and enzyme activities were determined in parallel.

**Vitality**

By measuring the chlorophyll content and PSII activities, we demonstrated that different Cd²⁺ concentrations are reflected by varying degrees of stress. We identified 5 µM Cd²⁺ as a tolerable condition, whereas 10 µM was found to lead to stress with loss of vitality. The highest Cd²⁺ concentrations were determined to be 70 µM Cd²⁺ in soils contaminated by Cd²⁺ (Lee, Lai & Chen 2004). Therefore, 10 µM Cd²⁺ is a value which might be encountered by *P. patens* in the environment. Cd²⁺ causes inhibition of chlorophyll biosynthesis, possibly by binding thiol groups of δ-aminolevulinic acid dehydratase at the active site as demonstrated for *Phaseolus vulgaris* (Padmaja, Prasad & Prasad 1990). Similarly, PSII activity of pea roots decreased significantly after a 2 h exposure of 0.1 and 1 mM Cd²⁺ (Balakhnina et al. 2005). Cd²⁺ influences photosynthesis at numerous sites (Mysliwa-Kurdziel, Prasad & Strzalka 2004). In detail, it causes damages of the light-harvesting chlorophyll *a/b* protein complex (Krupa 1988), PSII, chloroplast coupling factor 1 (Baszynski 1986; Balakhnina et al. 2005), the oxygen-evolving complex (van Duijvendijk-Matteoli & Desmet 1975), plastoquinones (Baszynski et al. 1980), as well as ferredoxin and ferredoxin NADP⁺ oxidoreductase (Siedlecka & Baszynski 1993). However, other studies examining Cd²⁺ effects on gene transcription of sulphate assimilation and GSH biosynthesis genes did not comprise investigations on vitality (Schäfer, Haag-Kerwer & Rausch 1998; Heiss et al. 1999; Harada et al. 2002; Sun et al. 2005b). Furthermore, it should be considered that previous results were obtained at very high Cd²⁺ concentrations [e.g. 25–400 µM, *A. thaliana* (ecotype Columbia, Xiang & Oliver 1998); up to 200 µM, *A. thaliana* (Harada et al. 2002; Sarry et al. 2006a); 100 µM, *F. antipyretica* and 19 other bryophyte species (Bruns et al. 2001); up to 100 µM, *Silene vulgaris* (van Hooft et al. 2001)]. Conversely, Cd²⁺ concentrations used in this study were much lower. Those lower concentrations are probably closer to what an organism might encounter in a contaminated environment and thus reflect a physiological adaptation towards Cd²⁺ exposure.

**Gene transcription of the assimilatory sulphate reduction pathway**

By real-time PCR, we identified the induction levels of genes encoding enzymes of cysteine and GSH biosynthesis. Our data for global enzyme expression agree with previous published results, but in this study, all participating enzymes were monitored. In the end, this yielded a more complete picture of the effects of Cd²⁺ exposure on *S.* metabolism in plants. In earlier studies, higher Cd²⁺ concentrations were applied, and the organisms investigated ranged from fungi to higher plants. Nevertheless, Cd²⁺ stress in *B. juncea* plants (25 µM) or *A. thaliana* (200 µM) caused transcripts of both ATPS and APR to be strongly up-regulated (Heiss et al. 1999) or 13-, 6- to 10-fold (Harada et al. 2002), respectively. Likewise, in *S. cerevisiae*, very high Cd²⁺ concentrations (1 mM) led to up-regulation of the MET3 gene (ATPS,
OAS-TL, O-acetylserine (thiol) lyase; γ-glutamyl cysteine (γEC) and glutathione (GSH) [nmol g⁻¹ fresh weight (FW)] in control- and Cd²⁺-stressed Physcomitrella patens after 24, 72 and 120 h of application

<table>
<thead>
<tr>
<th>Time after Cd²⁺ application (h)</th>
<th>Control</th>
<th>+5 µM CdCl₂</th>
<th>+10 µM CdCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Cysteine 22.7 ± 1.0</td>
<td>30.3 ± 3.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>33.9 ± 1.7&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>γEC 9.2 ± 0.7</td>
<td>8.2 ± 1.4</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>GSH 195.0 ± 13.2</td>
<td>226.2 ± 42.9</td>
<td>240.6 ± 10.6</td>
</tr>
<tr>
<td>72</td>
<td>Cysteine 24.9 ± 2.9</td>
<td>33.5 ± 6.3</td>
<td>41.5 ± 4.8&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>γEC 7.8 ± 0.2</td>
<td>10.8 ± 4.0</td>
<td>13.2 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>GSH 205.1 ± 3.6</td>
<td>286.9 ± 51.7</td>
<td>354.5 ± 27.0&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>Cysteine 18.2 ± 0.5</td>
<td>39.9 ± 8.5&lt;sup&gt;**&lt;/sup&gt;</td>
<td>49.4 ± 1.1&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>γEC 6.9 ± 0.5</td>
<td>14.4 ± 5.6</td>
<td>21.7 ± 2.0&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>GSH 191.1 ± 11.6</td>
<td>369.3 ± 44.1&lt;sup&gt;***&lt;/sup&gt;</td>
<td>425.5 ± 1.3&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

n = 3.
<sup>*</sup>P < 0.05; <sup>**</sup>P < 0.01; <sup>***</sup>P < 0.001.

Fauchon et al. 2002) and concomitantly, ATPS protein levels increased sixfold (Vido et al. 2001). In addition, a microarray analysis of S. cerevisiae under yet another Cd²⁺ concentration (300 µM CdCl₂) showed that MET14 (APK) and MET16 (PAPSR) had increased gene expression of 21- or sixfold (Momose & Iwahashi 2001).

In sharp contrast to other plants, where sulphate assimilation is only accomplished by APR, and PAPR is the initial step for the generation of O-sulphonated secondary compounds, P. patens is different. In this moss, two parallel pathways for sulphate assimilation coexist (Koprivova et al. 2002). Beside the plant-typical APR pathway, the PAPR pathway that is common for bacteria and fungi is also present. The presence of two pathways might be beneficial for the moss in meeting a higher demand for reduced sulphur under Cd stress.

For the regulation of the next step of sulphate reduction, conflicting data were previously published. Transcription of OAS-TL from P. patens is no different than that of B. juncea roots under similar conditions (25 µM CdNO₃). In both organisms, mRNA levels were only slightly increased (Schäfer et al. 1998). However, application of 50 µM Cd²⁺ to leaves of A. thaliana resulted in sevenfold induction in transcript levels of OAS-TL (Dominguez-Solis et al. 2001).

Sulphate assimilation is mainly regulated through the cysteine precursor OAS, which accumulates under sulphate starvation (Hirai et al. 2003; Wirtz, Droux & Hell 2004). OAS triggers the expression of several sulphate-reduction genes resulting in higher cysteine contents (Wirtz & Hell 2006). Subsequent synthesis of sulphur-containing defence compounds is needed to cope with biotic and abiotic stresses (Rausch & Wachter 2005). Nevertheless, transcription of the SAT gene, encoding the OAS-generating enzyme, was hardly influenced upon Cd²⁺ stress in P. patens. Increased sulphide demand [e.g. by stabilization of most likely intravacuolar GSH-complexed Cd(II) as described

<table>
<thead>
<tr>
<th>Time after Cd²⁺ application (h)</th>
<th>Enzyme activities</th>
<th>Control</th>
<th>+5 µM CdCl₂</th>
<th>+10 µM CdCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OAS-TL (U mg⁻¹protein)</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>γECS (mU mg⁻¹protein)</td>
<td>83.4 ± 7.1</td>
<td>81.0 ± 16.6</td>
<td>93.5 ± 8.9</td>
</tr>
<tr>
<td></td>
<td>GS (mU mg⁻¹protein)</td>
<td>400.1 ± 28.1</td>
<td>447.2 ± 6.0</td>
<td>484.1 ± 56.6</td>
</tr>
</tbody>
</table>

n = 3.
<sup>*</sup>P < 0.05; <sup>**</sup>P < 0.01.
OAS-TL, O-acetylserine (thiol) lyase; γ-ECS, γ-glutamyl cysteine synthetase; GS, GSH synthetase.
for high molecular weight (HMW) PC complexes in Schizosaccharomyces pombe, Clemens & Simm 2003] is accompanied by a higher OAS concentration which could explain the induction of sulphate assimilation genes as previously described.

Expression of genes encoding GSH biosynthesis enzymes

Multiple levels of regulation of sulphur metabolism and GSH homeostasis have been reported in numerous studies (reviewed by May et al. 1998; Leustek et al. 2000; Mendoza-Cózatl et al. 2005). A strong contribution of GSH as chelating agent for Cd²⁺ during cytoplasmic trafficking was shown for the moss F. antipyretica (Bruns et al. 2001). Up-regulation of γECS transcription is especially advantageous, because γECS is the key regulation step in GSH biosynthesis (Arisi et al. 1997; Mendoza-Cózatl & Moreno-Sanchez 2006). Generally, Cd²⁺ causes increased γECS mRNA in plants (Schäfer et al. 1998; Xiang & Oliver 1998; Dominguez-Solis et al. 2001). Analogously, in S. cerevisiae, the GSH1 gene (γECS) is up-regulated by 100 µM Cd²⁺ (Stephen & Jamieson 1997) while the protein amount is amplified 10-fold in the presence of 1 mM Cd²⁺ (Vido et al. 2001). Increased γECS transcription was determined in 30 µM Cd²⁺-treated Brassica napus, too (Sun et al. 2005b). In 200 µM Cd²⁺-stressed A. thaliana, levels of GS transcript coding for the last step of GSH biosynthesis were doubled (Harada et al. 2002).

While on the one hand GSH synthesis in Arabidopsis is driven by the GSH demand especially in response to stress situations (Meyer & Fricker 2002), on the other hand, GSH is known to act as repressor for cysteine biosynthesis in a feedback inhibition manner. In addition, sulphate assimilation is derepressed under sulphur-deficient conditions prevailing in heavy metal treated plants (Saito 2000).

Thiol levels

Using RP-HPLC, the levels of cysteine, γEC and GSH were determined upon Cd²⁺ exposure. After 24 h, Cd²⁺-stressed cultures contained higher cysteine pools than control plants. Because this increased cysteine concentration is neither reflected by mRNA levels of the respective biosynthesis genes nor by enhanced OAS-TL activity, cysteine is presumably released from protein breakdown during the first hours of Cd²⁺ stress. Alternatively, protein decay might not be required for increased cysteine supply because by analyzing the enzyme activities, the GSH biosynthesis rate appeared lower than the rate of the last step of cysteine synthesis. This might result in a higher steady-state cysteine concentration. Because the OAS-TL activity is higher than that of the enzymes of GSH biosynthesis, this step is probably not limiting. So overall, Cd²⁺ stress results in higher demands for cysteine and GSH, a fact that other studies have previously pronounced (Xiang & Oliver 1998; Dominguez-Solis et al. 2001).

Earlier findings suggest that bryophytes, which do not synthesize PCs, generally react upon Cd²⁺ stress by increased thiol pools (Bruns et al. 2001). We now have demonstrated that after adjustment of the metabolism to Cd²⁺, gene transcription and enzyme activity increased (γECS and GS). This could be identified as the underlying mechanisms that resulted in significant higher concentrations of cysteine and of GSH in these organisms.

Similarly, S. cerevisiae reacts to Cd²⁺ stress with a fourfold stimulation of GSH synthesis, too, as shown by [³⁵S]methionine pulse labelling (Vido et al. 2001). However, PC-biosynthesizing higher plants showed a decrease in the cellular GSH pool upon Cd²⁺ stress (Sarry et al. 2006b). This is caused by GSH demanding PC synthesis (Mendoza-Cózatl et al. 2005; Mendoza-Cózatl & Moreno-Sanchez 2006). For example, Cd²⁺-exposed Triticum aestivum exhibited higher PC biosynthesis, although the underlying PC calculation [(PC) = (non-protein thiols) – (GSH)] because of its oversimplification is probably questionable (Sun et al. 2005a).

In addition, in sieve tubes of B. napus, an increased level of GSH after Cd²⁺ treatment was reported (Nakamura et al. 2005).

Enzyme activities

Upon Cd²⁺ stress, we analysed enzyme activities of OAS-TL, γECS and GS in crude extracts of P. patens. Interestingly, OAS-TL assay results were not in accordance with real-time PCR results. Activity was decreased in the presence of Cd²⁺. Again, literature offers contradictory data for other plants. While a reduction of OAS-TL activity was observed in stressed maize plants (100 µM Cd²⁺) (Astolfi et al. 2004b), in Avena sativa (100 µM CdCl₂) or A. thaliana (50 µM CdCl₂), increased activities of OAS-TL were measured after Cd²⁺ application (Dominguez-Solis et al. 2001; Astolfi, Zuchi & Passera 2004a). However, total OAS-TL activity was much higher than that of the other enzymes of reductive S-metabolism. Therefore, OAS-TL is not the rate-limiting factor for the response to an elevated demand for thiols after Cd²⁺ exposure.

In addition, we investigated the activities of GSH biosynthesis enzymes, too. Considered to be the rate-limiting enzyme in GSH synthesis (Arisi et al. 1997), γECS should be elevated upon thiol-demanding stress situations. Our data showed that during Cd²⁺ stress, activities of γECS and GS were increased after 72 h. In the tomato cell line CdR6-0, which is capable of tolerating up to 0.3 mM CdCl₂, a twofold higher specific activity of γECS after 5 d was observed, while CdS cells, not able to tolerate this condition, exhibited the same GS activity (Chen & Goldsbrough 1994). Potentially γECS might be used to improve Cd²⁺ tolerance in plants. This has been shown for B. juncea over-expressing the E. coli gsh1 gene. Here, a fivefold higher γECS activity compared to wild-type B. juncea was observed that also resulted in increased Cd²⁺ tolerance (Zhu et al. 1999).

Recently, a theoretical attempt of modelling GSH and PC biosynthesis under Cd²⁺-stressed and non-stressed
conditions was published (Mendoza-Cózatl & Moreno-Sanchez 2006). In detail, GSH consumption (GSH-S-transferases, GSH peroxidases, PC synthase) was also taken into account. Applying this model to our results, we conclude that under Cd\(^{2+}\) stress, \(P.\) \textit{patens} uses the presumably most effective way to increase the GSH content. This is through enhanced γ-ECS activity.

In addition, the redox buffer GSH protects the cell against reactive oxygen species (ROS), which accumulate in response to biotic and abiotic stress (Mullineaux & Rausch 2005). In the ascorbate-GSH cycle, GSH reductase and GSH peroxidase link the function of GSH to that of ascorbic acid (Foyer & Noctor 2005; Noctor 2006). Moreover, GSH is consumed when it operates as a component of GSH S-transferase (GST)-based detoxification mechanisms for xenobiotics (Dixon, Davis & Edwards 2002).

Our findings agree with the hypothesis that Cd\(^{2+}\) detoxification is realized by intracellular chelation and sequestration through GSH. These mechanisms are especially important in bryophytes, which do not produce PC. Therefore, the GSH biosynthesis pathway was investigated step by step. And, for the first time, the different levels of potential regulation were investigated. In summary, it can be stated that the synthesis of cysteine upon Cd\(^{2+}\) stress is mainly regulated at the transcript level caused by varying OAS levels, while the biosynthesis of GSH is regulated on both, at the mRNA and enzyme activity level. We also investigated expression of the known ESTs by real-time PCR and sulphate uptake. However, preliminary data suggested that sulphate uptake was not altered upon Cd\(^{2+}\) exposure in \(P.\) \textit{patens} (data not shown). Further work will focus on transport, sequestration and intracellular localization of thiol peptides and their Cd\(^{2+}\) complexes in \(P.\) \textit{patens} cells to yield a complete picture of Cd\(^{2+}\) homeostasis in this plant.

ACKNOWLEDGMENTS

Helpful discussions with H. Tintemann, S. Menge and E. Püschel are gratefully acknowledged. This study was supported partially by the Deutsche Forschungsgemeinschaft (DFG) Graduate College ‘Adaptive physiological and biochemical Inorganic Chemistry’ and the Land Sachsen-Anhalt.

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