Analysis of Plant Pb Tolerance at Realistic Submicromolar Concentrations Demonstrates the Role of Phytochelatin Synthesis for Pb Detoxification

ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · MAY 2014

Impact Factor: 5.33 · DOI: 10.1021/es405234p · Source: PubMed

5 AUTHORS, INCLUDING:

Sina Fischer
University of Aberdeen
3 PUBLICATIONS **16** CITATIONS
SEE PROFILE

Tanja Kühnlenz
University of Bayreuth
3 PUBLICATIONS **9** CITATIONS
SEE PROFILE

Holger Schmidt
34 PUBLICATIONS **710** CITATIONS
SEE PROFILE

Stephan Clemens
University of Bayreuth
81 PUBLICATIONS **5,567** CITATIONS
SEE PROFILE

Environmental Science & Technology

Article

pubs.acs.org/est

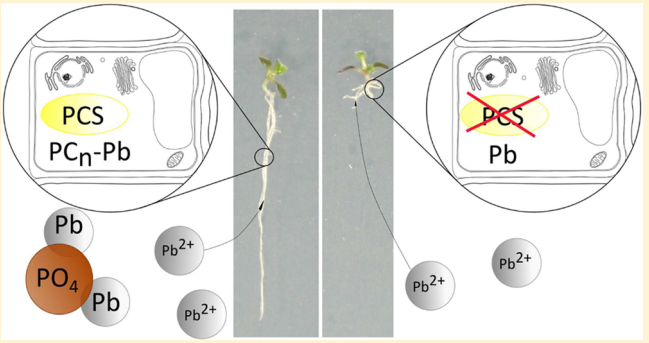
Analysis of Plant Pb Tolerance at Realistic Submicromolar Concentrations Demonstrates the Role of Phytochelatin Synthesis for Pb Detoxification

Sina Fischer,[†] Tanja Kühnlenz,[†] Michael Thieme,[†] Holger Schmidt,[†] and Stephan Clemens*[,†,‡]

[†]Department of Plant Physiology and [‡]Bayreuth Center of Ecology and Environmental Research, University of Bayreuth, Universitätsstrasse 30, 95440 Bayreuth, Germany

Supporting Information

ABSTRACT: Lead (Pb) ranks first among metals with respect to tonnage produced and released into the environment. It is highly toxic and therefore an important pollutant of worldwide concern. Plant Pb uptake, accumulation, and detoxification mobilize Pb into food webs. Still, knowledge about the underlying mechanisms is very limited. This is largely due to serious experimental challenges with respect to Pb availability. In most studies, Pb(II) concentrations in the millimolar range have been used even though the toxicity threshold is in the nanomolar range. We therefore developed a low-phosphate, low-pH assay system that is more realistic with respect to soil solution conditions. In this system the growth of *Arabidopsis thaliana* seedlings was significantly affected by the addition of only 0.1 μM $Pb(NO_3)_2$. Involvement of phytochelatins in the detoxification of Pb(II) could be demonstrated by investigating phytochelatin synthase mutants. They showed a stronger inhibition of root growth and a lack of Pb-activated phytochelatin synthesis. In contrast, other putative Pb hypersensitive mutants were unaffected under these conditions, further supporting the essential role of phytochelatins for Pb detoxification. Our findings demonstrate the need to monitor plant Pb responses at realistic concentrations under controlled conditions and provide a strategy to achieve this.



INTRODUCTION

Lead (Pb) is a soft, easy-to-process metal that has played an important role in human history due to these properties. Additionally, it was mined in ancient times because it occurs in geological formations in combination with silver. Pb was the first metal to be extracted from its ore, and since well before 3000 B.C. its processing has grown nearly exponentially.[1] In the course of production and utilization, Pb has ubiquitously been released into air, soil, and water, making it the first environmental pollutant.[1,2] Pb has no physiological function, and is highly toxic for plants and animals, and therefore a contaminant of global concern. In fact, Pb is the most toxic trace metal for plants with a toxicity threshold below 1 μM. Only mercury is nearly as toxic.[3]

Since the ban on leaded gasoline and other legislation aimed at restricting Pb pollution in the 1970s, a decline in atmospheric Pb can be noted. However, lead deposits in the environment continue to cause problems, keeping Pb in second place on the U.S. EPA's Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) priority list of hazardous substances (www.atsdr.cdc.gov/cercla/07list.html). Pb has serious effects on human health, especially for high risk groups such as infants, as recently discussed also by the European Food Safety Agency (EFSA).[4] Chronic low level exposure causes serious damage, most distinctly to the central nervous system, the vasculature, and the kidneys. To minimize the danger of Pb exposure for humans, food as the major source of exposure should ideally be kept free of Pb contamination. This requires an understanding of Pb(II) uptake and distribution systems in plants. Reported Pb concentrations of edible plant organs are on average 0.025 mg kg^{-1} (0.017 mg kg^{-1} in roots and tubers, 0.037 mg kg^{-1} in vegetables and cucurbits). In the range of 65−93% of the weekly dietary Pb intake of 2−4 μg kg^{-1} body weight stems from edible plant organs.[5]

Pb occurs in the environment mostly in inorganic form. The concentration in noncontaminated topsoils reaches on average 20 mg kg^{-1}. Close to sources of anthropogenic Pb emission it can increase to several thousand mg kg^{-1}.[4,6] Pb is highly persistent in soil. It has a low solubility at pH above 5[3,7−11] and occurs in the soil absorbed to particles such as Fe-, Al-, and Mn-oxides, as well as organic matter in highly stable complexes.[10] Also, Pb-phosphate precipitates form easily. Because of these characteristics, the bioavailability of Pb is extremely low, but can be strongly influenced by processes in the rhizosphere.

Received: November 24, 2013
Revised: April 7, 2014
Accepted: May 28, 2014

dx.doi.org/10.1021/es405234p | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Thus, ubiquitous Pb deposits can be locally mobilized into the food web via uptake into plants. This is a major reason for concern in light of the omnipresence of Pb. Depending on the soil contamination levels, Pb accumulation in above-ground organs of plants can vary.[12] Concentrations of subnanomolar to nanomolar are usually reached in soil solution at non-contaminated sites, and concentrations of 1−2 μM are reached at contaminated sites.[13] Any assay system used to study Pb(II) tolerance and accumulation should mimic the concentrations found under these natural conditions in order to enable a realistic assessment of plant responses to environmental Pb contaminations.

Early on it has therefore been proposed by several authors[14,15] that a tailor-made system is required to investigate Pb tolerance and detoxification mechanism in plants. Instead, most studies have resorted to applying up to millimolar concentrations to regular growth media[16−18] in order to trigger Pb toxicity effects. Such concentrations are about 3 orders of magnitude higher than the toxicity threshold.[3] They were employed due to the use of common growth media such as Murashige and Skoog (MS) or Hoagland, i.e. in the presence of high phosphate concentrations and at a pH > 5.

Possibly as a consequence of such experimental challenges, mechanistic understanding of plant Pb detoxification is rather limited. When applied at unrealistically high concentrations, most of the Pb remains extracellular, bound by or precipitated in the cell wall.[19−21]

The uptake into roots is hypothesized to occur through membrane transport proteins for essential metal ions. An example for such a hitchhiking of toxic metals on transporters with imperfect specificity is IRT1, which transports Fe(II) across the plasma membrane of root cells but also Cd(II).[22] In the case of Pb this assumption is, for instance, based on the observation that Pb toxicity could be ameliorated by addition of Ca(II) salts.[23] However, to date no transporter has been directly demonstrated to accept Pb(II) ions as substrate.

Once inside the plant, Pb(II) needs to be chelated cytosolically and sequestered in the vacuole, or transported out into the apoplast, where the extracellular immobilization of Pb clearly contributes to detoxification.[24] Glutathione and glutathione-derived phytochelatins (PCs) are thought to play a key role in complexing Pb(II).[25−27] PCs are peptides of the general structure $[\gamma\text{-Glu-Cys}]_n$-Gly ($n$ = 2−11). They are synthesized by phytochelatin synthases (PCS) in a trans-peptidase reaction. PCS is activated in vitro by the addition of various metal ions.[28,29] The initiation of PC production in Pb-exposed living plant tissue or algae has been shown several times, for instance for *Vetiveria zizanioides*,[30] *Salvinia minima*,[31] or *Chlamydomonas reinhardtii*.[32] However, the contribution of PC synthesis to Pb tolerance has not yet been demonstrated directly in loss-of-function experiments.

Our objectives were therefore two-fold: first, the development of a Pb tolerance assay system to study Pb effects under controlled conditions at realistic exposure levels that allow conclusions about the interaction of Pb with plants and the mobility of Pb in the environment; second, the analysis of PC formation and its role in Pb tolerance under such conditions.

EXPERIMENTAL SECTION

Plant Material and Cultivation Conditions. *Arabidopsis thaliana* wild-type (Col-0) and two *AtPCS1* mutant lines, *cad1-3*[33,34] and *cad1-6*,[35] were analyzed. For metal tolerance assays, seeds were surface-sterilized in chlorine gas atmosphere for 45 min. Five seeds per well were then placed in 5 mL of liquid medium in 6-well tissue culture plates containing either 1/10 strength Hoagland medium without microelements other than Fe (100 μM $(NH_4)_2HPO_4$, 200 μM $MgSO_4$, 280 μM $Ca(NO_3)_2$, 600 μM KNO_3, 5 μM Fe-HBED, 1% (w/v) sucrose, 5 mM MES, pH 5.7),[35] half-strength MS medium from Duchefa (10.30 mM NH_4NO_3, 9.38 mM KNO_3, 1.49 mM $CaCl_2$, 0.75 mM $MgSO_4$, 0.62 mM KH_2PO_4, 49.93 μM NaFeEDTA, 50.17 μM H_3BO_3, 49.98 μM $MnSO_4$, 26.57 μM $ZnSO_4$, 2.49 μM KI, 0.52 μM Na_2MoO_4, 0.08 μM $CuSO_4$, 0.10 μM $CoCl_2$, 13.30 μM glycine, 0.28 mM myo-inositol, 2.04 μM nicotinic acid, 1.48 μM pyridoxine, 0.15 μM thiamine, pH 5.7), or a low-phosphate/low-pH (LPP) medium (10 μM $(NH_4)_2HPO_4$, 90 μM NH_4NO_3, 200 μM $MgSO_4$, 280 μM $Ca(NO_3)_2$, 600 μM KNO_3, 5 μM Fe-HBED, 1% (w/v) sucrose, 5 mM MES, pH 5.0). MES solution (Sigma-Aldrich) was sterile-filtered according to the manufacturer's guidelines. After stratification for 2 d at 4 °C, the plates were incubated at 22 °C for 9 d under long-day conditions (16 h light/8 h dark) and moderate shaking (80 rpm). The medium was exchanged at days 6 and 8, and pooled for pH determination and ICP analysis. At day 9 the root length of each seedling was measured.

For PC analysis and metal accumulation assays, plants were grown in the same media in 50-mL Falcon tubes to obtain more fresh weight.

Inductively Coupled Plasma−Optical Emission Spectroscopy. Pb accumulation in seedlings was determined after washing with H_2O (twice, 10 min), 100 mM $CaCl_2$ (once, 10 min), and 50 mM EDTA (twice, 5 min). All washing steps were performed at 4 °C. Dry plant material was digested in 2 mL of HNO_3 and 1 mL of H_2O_2 for 12 min at 180 °C using a microwave (Start 1500, MLS GmbH). For the analysis of available Pb, nutrient solutions were centrifuged for 30 min at 2900*g*, 20 °C. Six mL of supernatant was sampled. Metal concentrations were measured via ICP−OES (ICAP 6500, Thermo): Pb at 220.3 nm, Zn at 213.8 nm, Fe at 238.2 nm, and Mn at 257.6 nm.

AtPCS1 Recombinant Protein Production. *E. coli* BL21-AI cells were used to express AtPCS1 recombinantly. The vector pJC40 was modified to carry the *AtPCS1* cDNA in frame with an N-terminal His-tag (6xHis-AtPCS1). Protein expression was induced at an OD_{600} of 0.8 by addition of arabinose to a final concentration of 0.2% (w/v). For 4 h the culture was incubated at 21 °C and 200 rpm, after which the cells were harvested (4 °C, 18 400 g, 25 min). The pellet was washed with 20 mL of water, and cells were lysed for 30 min at 4 °C by addition of 3 mL of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) per gram pellet and 1 mg lysozyme mL^{-1}. Cells were further disrupted by sonication in 6 intervals (10 s treatment, 10 s cooling on ice). After centrifugation (25 min, 6160*g*, 4 °C), the soluble protein was bound to Ni-NTA (Qiagen) matrix (4:1) for 1 h at 4 °C. Following two washing steps (4 mL of 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) the protein was eluted four times by addition of 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8). Each fraction was collected separately.

PCS Activity Assay. To test for 6xHis-AtPCS1 activity, 80 μL of activity buffer (50 mM HEPES pH 7.0, 12.5 mM GSH, 10% (v/v) glycerol) was added to heterologously expressed protein in 20 μL of storage buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7.0, 50% (v/v) glycerol). The

enzyme was activated by addition of either Cd(II) or Pb(II). The reaction was carried out for 30 min at 35 °C and stopped by addition of 1.1 μL 10% (v/v) TFA. Thiols were derivatized with monobromobimane at 37 °C for 30 min in the dark as described previously.[35]

PC Quantification. Five μL of the labeled reaction products was injected and analyzed via HPLC (Reprosil 100 C18, 5-μm particle size, 250-mm length, 4.6-mm interior diameter, X-LC Jasco). The column was developed with a linear binary gradient of water (solvent A) and 95% (v/v) acetonitrile (solvent B), both acidified with 0.1% TFA at a flow of 1 mL min^{-1}: 12% B, in 9 min to 25% B, in 4 min to 35% B, in 3.5 min to 90% B. The temperature of the column was 40 °C. Monobromobimane-labeled thiols were detected with a fluorescence detector (excitation wavelength 380 nm, emission monitored at 480 nm, FP-2020_{Plus} Jasco). For quantification of PC2 to PC5, PC standards (AnaSpec) were labeled accordingly with the final concentrations of 25, 50, 100, and 200 μM.

For the sake of higher sensitivity, PCs in plant tissue were quantified via Ultra performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS). Using this technique the limit of PC2 detection in our setup was about 50 fmol as compared to around 200 pmol for HPLC and fluorescence detection. Ground plant material was frozen in liquid N_2 followed by extraction of the PCs with TFA (0.1%) and 6.3 mM DTPA (300 μL per 100 mg). UPLC-ESI-QTOF-MS analyses were performed as described previously.[36]

RESULTS AND DISCUSSION

Pb Availability and Toxicity in Standard Growth Media. *AtPCS1* encodes the major PCS in *A. thaliana*. Its importance for survival of plants under conditions of excess Cd, As, and Zn is well-documented.[34,35] A role in Pb detoxification has been suggested but not demonstrated. We therefore decided to develop a Pb tolerance assay along the question as to whether PC synthesis contributes to Pb detoxification. We assayed growth responses of the two *AtPCS1* mutants *cad1-3*[33] and *cad1-6*,[35] as well as the respective wild-type Col-0 in liquid seedling assays. First, we evaluated commonly used growth media, namely 1/2 MS and 1/10 Hoagland medium. In accordance with the recommendations for metal phytotoxicity studies in solution culture by Kopittke et al.,[3] we monitored critical medium characteristics, i.e. pH and soluble Pb concentration.

When $Pb(NO_3)_2$ solution was added to 1/2 MS medium, white Pb precipitates formed immediately that drastically reduced bioavailable Pb. To document differences in Pb solubility, the media were analyzed via ICP−OES and the soluble Pb concentration was determined. In medium containing between 1 and 1000 μM Pb(II), the recovery of soluble Pb was below 1.5%, regardless of the presence or absence of seedlings (Figure 1C; for absolute concentrations of soluble Pb(II) please refer to Table S1 in the Supporting Information(SI)). With the exception of the two lowest Pb(II) concentrations, the measured concentrations of available Pb(II) were in good agreement with theoretical values determined with the metal speciation software Visual MINTEQ (http://vminteqlwr.kth.se/) (SI Table S1). At 10 mM Pb, about 80% was recovered (ca. 90% according to Visual MINTEQ speciation), indicating that PO_4^{3-} had been nearly completely precipitated (>90% according to calculation, SI Table S1), leaving the residual Pb soluble. Furthermore, the higher the

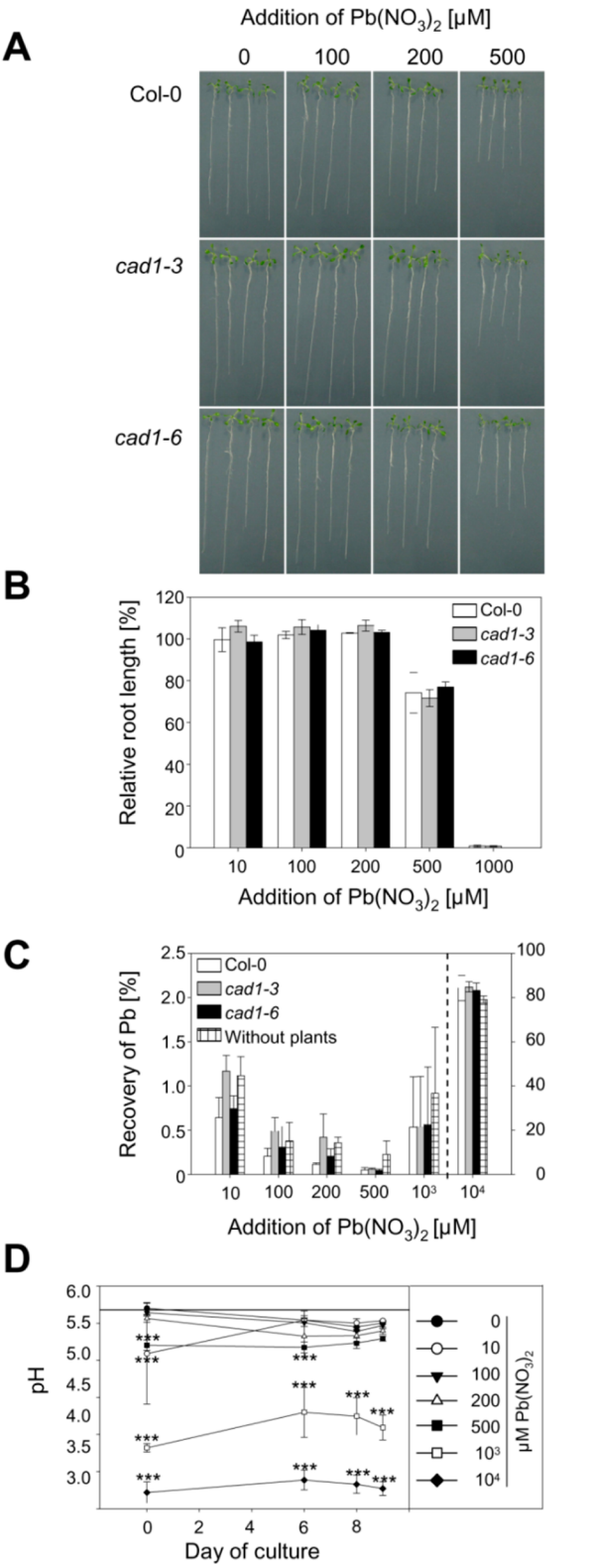


Figure 1. Pb tolerance of *A. thaliana* Col-0 wild type and *cad1* mutant seedlings (*cad1-3* and *cad1-6*) in 1/2 MS medium. (A) Plants cultivated in the liquid system for 9 d in the presence of increasing Pb(II) concentrations were photographed after placing them on agar plates. (B) The relative root length was calculated as % of the growth under control conditions. Data represent 2 independent experiments (n = 16−45 seedlings total); shown are mean values ± SD. Mean root length (± SD) under control conditions: Col-0: 62 ± 6.9 mm; *cad1-3*: 59 ± 8 mm; *cad1-6*: 61 ± 6.2 mm. (C) Recovery of soluble Pb from

dx.doi.org/10.1021/es405234p | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Figure 1. continued

the growth medium as determined by ICP−OES after centrifugation. The recovery for medium spiked with 10 mM $Pb(NO_3)_2$ is indicated on the right axis. Shown are mean values ± SD, n = 3−6. (D) Monitoring of pH during cultivation and in the absence of seedlings. The horizontal line represents the initial pH of 5.7, adjusted after autoclaving. Shown are mean values ± SD, n = 3. Asterisks indicate significant differences (determined via two-way Anova, Tukey test) to control conditions; ***: $P < 0.001$.

intended Pb stress, the more precipitate formed and the greater the effects on other parameters, most notably the pH of the medium, which was strongly affected by the addition of $Pb(NO_3)_2$ in a concentration-dependent manner (Figure 1D).

Seedlings of the three genotypes showed equal growth in 1/2 MS medium under control conditions. Addition of 500 μM Pb(II) was required to achieve a significant reduction in root growth by about 40% (Figure 1A, B). Under these conditions only 0.2% (1.13 μM) of the added Pb remained soluble. A concentration of 1 mM Pb(II) led to a growth reduction of 97% (Figure 1B). Seedlings did not survive. Across all Pb(II) concentrations applied, no difference in growth between the wild-type and the two *cad1* mutants could be observed.

The inhibitory concentrations are in agreement with those of previous studies employing 1/2 MS medium. For instance, *A. thaliana EIN2*,[37] *PEN3*,[38] and *PDR12*[39] were implicated in *A. thaliana* Pb tolerance based on growth inhibition in the presence of 300−750 μM Pb(II). This similarity in concentrations needed to achieve Pb toxicity indicated that the liquid seedling system we used is suitable for developing Pb tolerance assays aiming to determine Pb effects at more realistic concentrations. However, the monitoring of medium conditions also revealed that only a minute fraction of the added Pb(II) is available in this widely used medium. Moreover, massive side effects such as a lowering of pH and nearly complete precipitation of phosphate occur, raising the possibility that factors other than Pb toxicity account for reported growth phenotypes.

Only 0.2% of the Pb was available in 1/2 MS when applied at a concentration of 500 μM (Figure 1C). This medium contains 624 μM phosphate, whereas in a soil solution phosphate concentrations range from 5−45 μM.[6,40] The ionic strength of the 1/10 Hoagland medium is much lower and thus closer to the recommended use of dilute media.[3] Phosphate concentration is reduced to 100 μM. Here the addition of 100 μM Pb(II) was sufficient to achieve an inhibitory effect on the growth of Col-0 (Figure 2 and SI Figure S1A). This time the *cad1* mutants showed higher sensitivity to Pb(II) than the wild-type, indicating a direct contribution of PC synthesis to Pb detoxification. After 10 and 100 μM Pb(II) addition, *cad1-3* and *cad1-6* exhibited relative root growth of about 70% and 40%, which represents a significant reduction relative to Col-0 (ca. 90% and 75%, respectively). Importantly, the appearance of a Pb(II) hypersensitivity phenotype of the *cad1* mutants in 1/10 Hoagland medium demonstrated that 1/2 MS medium is not only unsuitable for assaying Pb tolerance under controlled conditions but can even mask respective phenotypes.

The synthesis of PCs has many times been implicated in Pb tolerance.[26,29,41,42] Pb-elicited PC accumulation[31,32,42,43] and the formation of PC−Pb complexes[30,32,41,44] are documented in various terrestrial plants and in algae. Direct genetic evidence for the importance of PC synthesis, however, cannot be

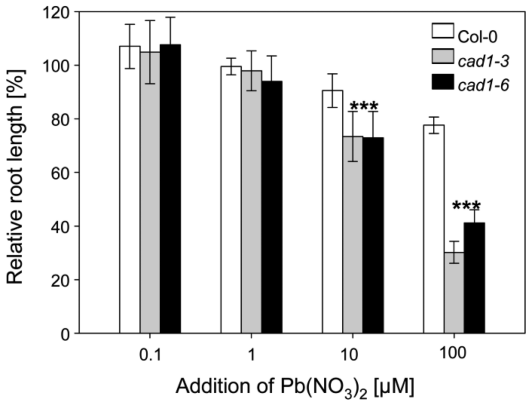


Figure 2. Pb tolerance of *A. thaliana* Col-0 wild type and *cad1* mutant seedlings (*cad1-3* and *cad1-6*) in 1/10 Hoagland medium. The relative root length of seedlings cultivated in the presence of increasing Pb(II) concentrations is shown. It was calculated as percent of the growth under control conditions. Data represent 3 independent experiments (n = 41−72 seedlings total); shown are mean values ± SD. Significant differences to the wild-type were determined by a two-way Anova, Tukey test; ***: $p < 0.001$. Mean root length (± SD) under control conditions: Col-0: 61.2 ± 7.9 mm, *cad1-3*: 61.5 ± 5.5 mm, *cad1-6*: 64.2 ± 8.1 mm.

obtained in the most widely used MS medium as shown in Figure 1, whereas growth assays in 1/10 Hoagland medium revealed a Pb hypersensitivity phenotype of *cad1* mutants.

Although 1/10 Hoagland is more dilute than 1/2 MS, Pb solubility was nonetheless very limited. At 0.1 and 1 μM Pb(II), around 10 and 5% of the added Pb were recovered (0.01 and 0.05 μM), respectively, in medium without plants. At 10 and 100 μM Pb(II) the recovery decreased to approximately 0.5%, and to 0.1% in medium without plants (SI Figure S1B and Table S1). Again, for the higher Pb(II) concentrations the measured concentrations agreed well with theoretical values according to Visual MINTEQ (SI Table S1). In the presence of seedlings Pb was consistently more bioavailable, indicating solubilizing activities at the root surface. The pH of the medium showed an increase over the course of the experiment from 5.7 to 5.8 (SI Figure S1C).

A New Assay System to Assess Pb Tolerance at Environmentally Relevant Concentrations. Corresponding to the low Pb availability, the Pb(II) concentration of 100 μM required for about half-maximal growth inhibition of wild-type roots in 1/10 Hoagland medium was still 2 orders of magnitude higher than the actual toxicity threshold for Pb and the concentration that can occur in soil solutions of metal-toxic soil.[3] Therefore, we reduced phosphate concentrations even further to 10 μM. In addition, the pH of the medium was lowered from 5.7 to 5.0, the threshold above which Pb solubility drops sharply.[3] Monitoring of pH during plant growth showed increases after day 6 of cultivation. Therefore, medium was exchanged at days 6 and 8 of cultivation to maintain the pH at 5.0.

In spite of the suboptimal pH and phosphate supply, seedlings developed well in the new medium yet with slower growth rates. After 9 days under control conditions the roots of Col-0, *cad1-3*, and *cad1-6* reached root lengths of 44.5 ± 7.9, 49.2 ± 9.9, and 51.6 ± 5.4 mm, respectively. Thus, no differences between wild type and *cad1* mutant seedlings were observed in the absence of Pb(II), indicating that PC deficiency

dx.doi.org/10.1021/es405234p | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

did not render seedlings more sensitive to the suboptimal growth conditions of the LPP medium.

We then assayed Pb tolerance of the wild-type and the two *cad1* mutants. Toxic effects of Pb exposure were already detectable upon the addition of 0.05 μM Pb(II) (Figure 3 and

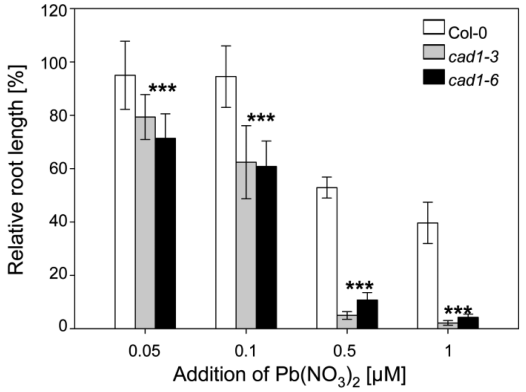


Figure 3. Pb tolerance of *A. thaliana* Col-0 wild type and *cad1* mutant seedlings (*cad1-3* and *cad1-6*) in the low-phosphate/low-pH medium. The relative root length of seedlings cultivated in the presence of increasing Pb(II) concentrations is shown, calculated as % of the growth under control conditions. Data represent 3 independent experiments (n = 16−56 seedlings total); shown are mean values ± SD. Significant differences from the wild-type were determined by a two-way Anova, Tukey test; ***: $p < 0.001$. Mean root length (± SD) under control conditions: Col-0: 44.5 ± 7.9, *cad1-3*: 49.2 ± 9.9, *cad1-6*: 51.6 ± 5.4 mm.

SI Figure S2A). Col-0 root growth was reduced by ca. 10%. The difference in growth of the wild-type in comparison to the growth at control conditions became significant at 0.1 μM Pb(II). In the presence of 0.5 μM Pb(II), Col-0 roots reached less than 60% of control growth. This strong increase in Pb sensitivity correlated with higher Pb availability, which was close to or greater than 20% in the presence of seedlings under all conditions (SI Figure S2B and Table S1). In medium without seedlings, we found availabilities of >60% while according to Visual MINTEQ all Pb(II) should have been available (SI Table S1).

The effect of Pb exposure on root growth of *cad1-3* and *cad1-6* seedlings was significantly more pronounced than that on wild-type seedlings at all tested concentrations (Figure 3). The two *cad1* mutants did not show a distinct difference in their response toward Pb(II). The difference between Col-0 and *cad1* mutants was strongest at the two highest Pb(II) concentrations where the root length was reduced by more than 90%. Approximately half-maximal inhibition was observed at 0.1 μM Pb(II) as compared to ca. 0.5 μM Pb(II) for Col-0. These differences between the PC deficient mutants and their wild-type again strongly suggested a role of PC synthesis in the detoxification of Pb.

For reference we also tested Cd sensitivity of Col-0 and *cad1-3* seedlings in the newly established medium. Addition of 0.5 μM Cd(II) inhibited wild-type root growth by about 65%. The well-known Cd hypersensitivity of *cad1-3* seedlings was apparent at all tested concentrations (SI Figure S3).

We also tested suitability of the three media for Pb bioaccumulation assays. Two Pb(II) concentrations each were chosen: the highest concentration not inhibiting growth and the lowest growth-inhibitory concentration. When relative Pb accumulation was determined as % Pb accumulated relative to Pb(II) added to the medium, dramatic differences between the media were found, which corresponded well with the results of the tolerance assays. Values ranged from 0.005 to 0.03% in 1/2 MS medium, from 0.1 to 0.6% in 1/10 Hoagland medium, and from 7 to 10% in LPP medium (SI Figure S4A). Higher apparent absolute accumulation in 1/2 MS medium might indicate desorption problems caused by precipitation due to the massive concentrations of Pb(II) added to the assay (SI Figure S4B). No significant genotype-dependent differences were found under any of the tested conditions, suggesting that the Pb(II) hypersensitivity of *cad1* mutants is not associated with altered Pb accumulation.

In addition we determined contents of the main micronutrients Zn, Fe, and Mn. They were consistently higher for 1/2 MS and very similar for 1/10 Hoagland and the LPP medium (SI Figure S5). Importantly, these data show that the slower growth in LPP medium does not result from micronutrient deficiencies since levels were as high as in the 1/10 Hoagland medium that supported growth rates equal to those in 1/2 MS medium under control conditions. Moreover, no differences between genotypes were observed arguing once more against a general hypersensitivity of *cad1* mutants in the LPP medium.

Taken together, these data demonstrated the suitability of the LPP medium for assaying Pb tolerance at available Pb concentrations that mimic those occurring in acidic soils and at contaminated sites, i.e. under conditions that are most likely to mobilize soil Pb deposits into the food web.[45,46] Furthermore, tolerance toward other toxic metals such as Cd can be reliably assessed, too.

Pb-Activated PC Synthesis. To test whether the apparent contribution of AtPCS1 to Pb detoxification is associated with Pb-elicited PC synthesis, control and Pb(II)-exposed seedlings were analyzed for their thiol content. Shown in Figure 4A are the PC2 concentrations found in whole seedlings after incubation with 1 μM $Pb(NO_3)_2$ for 3 days. An increase in PC2 levels upon Pb exposure could be detected only in Col-0 wild-type, while the mutants *cad1-3* and *cad1-6* did not accumulate more PC2 in the presence of Pb(II) than the background levels measurable in control seedlings. Thus, Pb-elicited PC formation was detected in Col-0 seedlings cultivated in the presence of a Pb(II) concentration that was 700- to 3000-fold lower than in previous cell culture[26,47] or whole plant studies.[30,42,43] Background PC2 levels in *cad1-6* mutant seedlings have been observed before.[35] They might be attributable to low but intact AtPCS2 activity in combination with residual AtPCS1 activity.

PC synthesis is elicited through direct activation of the constitutively expressed PCS protein by metal ions or GS-metal complexes.[48,49] Thus, we asked whether purified AtPCS1 can be activated by Pb(II) ions in vitro. For this purpose 6x-His-AtPCS1 was expressed in *E. coli* and affinity-purified (Figure 4B). The eluted protein was used for in vitro activity assays. We aimed to directly compare Pb(II) effects to those of Cd(II) ions, which are well-known to be strongly activating. Therefore, pH of the assay system was lowered and Pb(II) availability was again estimated by ICP−OES analysis. According to these tests, 20% more Pb(II) was applied to compensate for the amount that precipitated. Pb(II) ions were indeed found to elicit PC formation at all three concentrations tested. Activation relative to the corresponding Cd(II) concentration ranged from about 25 to about 50% (Figure 4C). Although it is not possible to accurately determine Pb and Cd concentrations available for enzyme activation because, for instance, it is not clear whether

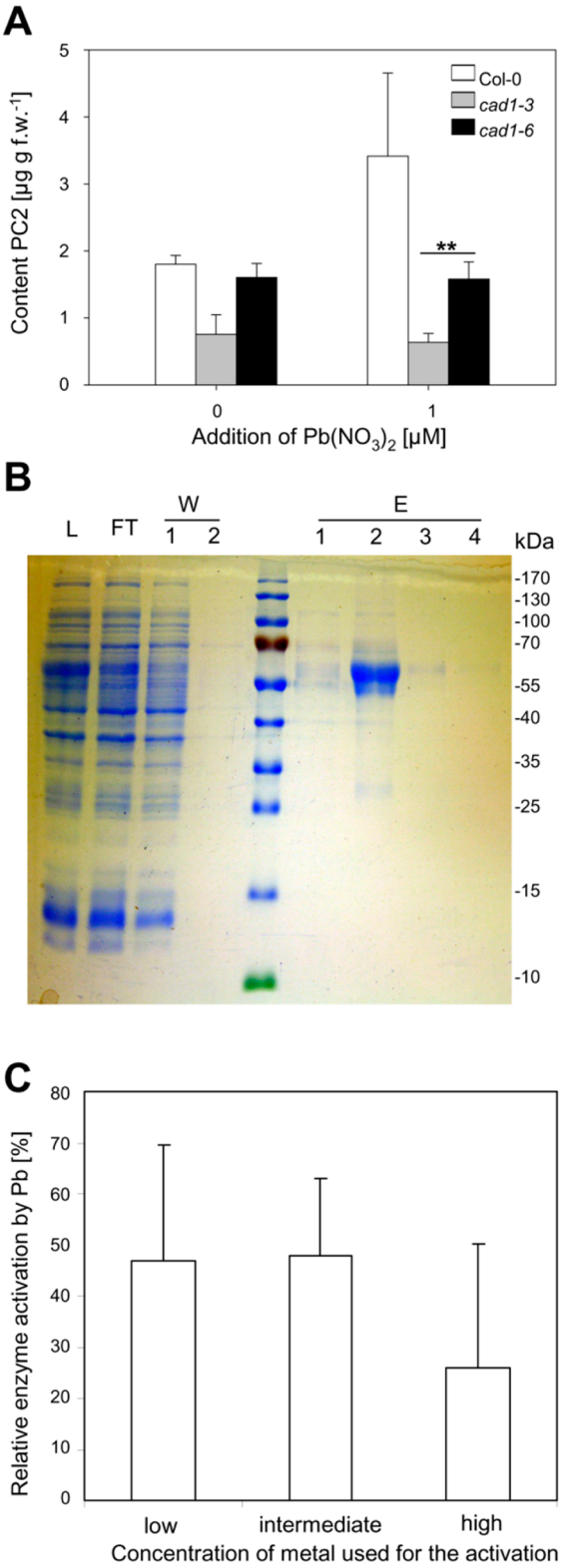


Figure 4. Phytochelatin synthase *At*PCS1 is activated by Pb in vivo and in vitro. (A) PC2 accumulation is increased in Pb(II)-treated *A. thaliana* Col-0 wild-type seedlings, but not in *cad1* mutant seedlings (*cad1-3* and *cad1-6*). One-week-old plants were incubated for 3 d in low-phosphate/low-pH medium containing 0 or 1 μM Pb(II). Following thiol extraction and labeling with monobromobimane, PC2 concentrations were determined via UPLC-ESI-QTOF-MS. Shown are mean values ± SD (n = 3−4). Significant differences between the mutants and the wild-type were determined by a two-way Anova, Tukey test; **: $p < 0.01$. (B, C) Recombinant purified 6x-His-AtPCS1 is activated by Pb(II). Documented in (B) is the affinity-purification of 6x-His-AtPCS1 (size ca. 58 kDa). Shown are bacterial lysate (L), flow through (FT), wash (W), and eluates (E). (C) Purified 6x-His-AtPCS1 was assayed for activity in the presence of Pb(II). Activation of PCS activity by Pb(II) was compared to that by strongly activating Cd(II) ions. Activities were compared at low, intermediate, and high metal ion concentrations with compensation for differences in solubility in the assay buffer: low: 1 μM Cd(II) or 1.2 μM Pb(II); intermediate: 10 μM Cd(II) or 12 μM Pb(II); high: 100 μM Cd(II) or 120 μM Pb(II). Three independent 6x-His-AtPCS1 purifications were assayed. Activities are expressed relative to the corresponding Cd(II) concentration. Error bars indicate SD.

hydrated ions or GS-metal complexes activate PCS,[48,49] these results still demonstrate efficient activation of PC synthesis by Pb.

Evaluation of Reported Pb Hypersensitivity Phenotypes. Having established PC synthesis as an essential component of plant Pb detoxification for *A. thaliana* seedlings, we decided to assess the importance of this process relative to other proposed Pb detoxification mechanisms. We therefore initiated the re-evaluation of *A. thaliana* mutants previously reported to be Pb hypersensitive by testing them at the more realistic Pb(II) concentrations of our LPP medium. These mutants included the ethylene signaling mutant *ein2*[37] and another ethylene insensitive line (*etr1*), as well as two different mutant alleles of the ABC transporter PEN3.[50] As shown in Figure 5, none of these mutants displayed any Pb hypersensitivity compared to wild-type seedlings while the strong Pb hypersensitivity of *cad1-3* seedlings grown alongside the other mutants was again apparent.

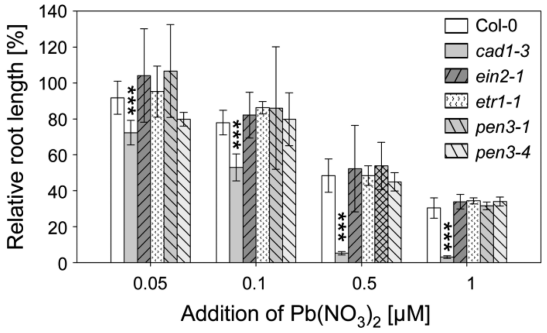


Figure 5. Mutants previously reported as Pb hypersensitive show wild-type growth responses to toxic Pb(II) concentrations in the LPP medium. Relative root length of seedlings cultivated in the presence of increasing Pb(II) concentrations is shown. It was calculated as % of the growth under control conditions. Data represent 2−9 independent experiments (n = 10−134 seedlings total); shown are mean values ± SD. Significant differences from the wild-type were determined by a two-way Anova, Tukey test; ***: $p < 0.001$. Mean root lengths (± SD) under control conditions were Col-0: 39.9 ± 8.8, *cad1-3*: 45.7 ± 9.2, *ein2-1*: 37.2 ± 15.9, *etr1-1*: 45.5 ± 9.4, *pen3-1*: 42.4 ± 13, and *pen3-4*: 46.3 ± 7.7 mm.

This finding emphasizes the need to assess Pb tolerance in a relevant concentration range and under conditions that allow control over available Pb. Plant Pb tolerance mechanisms proposed previously based on loss-of-function mutants beyond the ones tested here, as well as gain-of-function transgenic overexpressing lines, should be re-evaluated accordingly. The assessment of assay conditions in 1/2 MS medium clearly

dx.doi.org/10.1021/es405234p | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

demonstrated that a strong decrease in pH and severe phosphate deficiency develop, which might well explain the previously reported growth phenotypes in the presence of Pb(II). Likewise, such re-evaluation in LPP medium should include other potential Pb detoxification pathways such as cell wall modifications. Pb can be immobilized by pectins.[21] Similarly, callose depositions were suggested to act as barriers for Pb uptake in *Lemna minor* L.[51] and *A. thaliana*.[51,52]

Furthermore, the LPP conditions should be used to study plant Pb accumulation. It is only under such conditions that appreciable uptake into plants is likely to occur naturally. Possible problems due to phosphate starvation after prolonged growth could be circumvented by restricting the periods of Pb exposure.

In conclusion, our data directly demonstrate an important role for Pb-activated phytochelatin synthesis in Pb detoxification at the seedling stage. This cannot be shown in the plant growth medium most widely used for stress tolerance studies, while the LPP medium we established allows control over Pb availability and the monitoring of plant responses to Pb exposure at environmentally relevant concentrations. *A. thaliana* wild-type seedlings showed clear Pb toxicity effects already at 0.1 μM Pb(II), i.e. at a concentration 3 orders of magnitude lower than in most published studies.

As suggested by Kopittke et al.[3] for metal phytotoxicity studies in solution culture, our assay mimics phosphate concentrations in soil solutions, reduces interference by other minerals, and documents the pH over the course of plant growth. Furthermore, we measured and reported the actual available Pb in the solution to which the plants were subjected. Growth assays showed Pb hypersensitivity of *A. thaliana cad1* mutants with a defect in the *AtPCS1* gene, a lack of Pb-elicited PC2 synthesis in these mutants, and activation of recombinant purified 6xHis-AtPCS1 by Pb(II). In contrast, mutants previously reported as Pb hypersensitive did not show any growth defects relative to wild-type in the presence of toxic Pb(II) concentrations in our assays. This further emphasizes the need to analyze the interaction of Pb with plants at environmentally relevant concentrations and under conditions that allow control of Pb availability. Such studies will enable a better understanding of a major pathway for the mobilization of environmental Pb deposits into the food web. Such mechanistic insights have in addition the potential to advance phytoremediation of Pb pollution.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1: Evaluation of the Pb tolerance assay in 1/10 Hoagland medium. Figure S2: Evaluation of the Pb tolerance assay in low phosphate/low pH medium. Figure S3: Cd hypersensitivity of *cad1*-3 mutants in low phosphate/low pH medium. Figure S4: Comparison of the Pb bioaccumulation of Col-0 and the two cad1 mutants in the three described media. Figure S5: Accumulation of the main micronutrients Fe, Zn, and Mn in *A. thaliana* seedlings cultivated in the three different media. Table S1: Comparison of experimentally determined and calculated Pb fractions availability in the different growth media used to test plant Pb tolerance. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: stephan.clemens@uni-bayreuth.de; tel: 49-921-552630; fax: 49-921-552642.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge financial support by the University of Bayreuth and in part by the Deutsche Forschungsgemeinschaft (DFG CL152/7-1).

■ REFERENCES

(1) Nriagu, J. O. A history of global metal pollution. *Science* **1996**, *272* (5259), 223−223.
(2) Nriagu, J. O. Tales told in lead. *Science* **1998**, *281* (5383), 1622−1623.
(3) Kopittke, P. M.; Blamey, F. P. C.; Asher, C. J.; Menzies, N. W. Trace metal phytotoxicity in solution culture: A review. *J. Exp. Bot.* **2010**, *61* (4), 945−954.
(4) EFSA. Panel EFSA - Scientific opinion of the CONTAM Panel: Lead in food. *EFSA J.* **2010**, *8* (4), 1570−1717.
(5) WHO 976. Lead (WHO Food Additives Series 44) http://www.inchem.org/documents/jecfa/jecmono/v44jec12.htm (accessed Aug 14, 2012).
(6) Parker, D. R.; Norvell, W. A. Advances in solution culture methods for plant mineral nutrition research. *Adv. Agron.* **1999**, *65*, 151−213.
(7) Wong, M. H.; Bradshaw, A. D. A Comparison of the toxicity of heavy metals, using root elongation of rye grass *Lolium perenne*. *New Phytol.* **1982**, *91* (2), 255−261.
(8) Weng, L.; Temminghoff, E. J. M.; Van Riemsdijk, W. H. Determination of the free ion concentration of trace metals in soil solution using a soil column donnan membrane technique. *Eur. J. Soil Sci.* **2001**, *52* (4), 629−637.
(9) Martínez, C.; Motto, H. Solubility of lead, zinc and copper added to mineral soils. *Environ. Pollut.* **2000**, *107* (1), 153−158.
(10) Lee, S.-Z.; Chang, L.; Yang, H.-H.; Chen, C.-M.; Liu, M.-C. Adsorption characteristics of lead onto soils. *J. Hazard. Mater.* **1998**, *63* (1), 37−49.
(11) Sukreeyapongse, O.; Holm, P. E.; Strobel, B. W.; Panichsakpatana, S.; Magid, J.; Hansen, H. C. B. pH-dependent release of cadmium, copper, and lead from natural and sludge-amended soils. *J. Environ. Qual.* **2002**, *31* (6), 1901.
(12) Bovenkamp, G. L.; Prange, A.; Schumacher, W.; Ham, K.; Smith, A. P.; Hormes, J. Lead uptake in diverse plant families: A study applying X-ray absorption near edge spectroscopy. *Environ. Sci. Technol.* **2013**, *47* (9), 4375−4382.
(13) McLaughlin, M.; Parker, D.; Clarke, J. Metals and micronutrients − Food safety issues. *Field Crops Res.* **1999**, *60* (1−2), 143−163.
(14) Påhlsson, A.-M. B. Toxicity of heavy metals (Zn, Cu, Cd, Pb) to vascular plants. *Water, Air, Soil Pollut.* **1989**, *47* (3), 287−319.
(15) Godbold, D. L.; Hüttermann, A. The uptake and toxicity of mercury and lead to spruce (*Picea abies* Karst.) seedlings. *Water, Air, Soil Pollut.* **1986**, *31* (1), 509−515.
(16) Kim, D.-Y.; Bovet, L.; Kushnir, S.; Noh, E. W.; Martinoia, E.; Lee, Y. AtATM3 is involved in heavy metal resistance in Arabidopsis. *Plant Physiol.* **2006**, *140* (3), 922−932.
(17) Wang, Y.; Zong, K.; Jiang, L.; Sun, J.; Ren, Y.; Sun, Z.; Wen, C.; Chen, X.; Cao, S. Characterization of an Arabidopsis cadmium-resistant mutant cdr3-1D reveals a link between heavy metal resistance as well as seed development and flowering. *Planta* **2011**, *233* (4), 697−706.
(18) Yu, B.; Bian, X.; Qian, J.; Chen, X.; Wang, R.; Cao, S. Arabidopsis desaturase 2 gene is involved in the regulation of cadmium and lead resistance. *Plant Soil* **2012**, *358* (1−2), 289−300.

(19) Fahr, M.; Laplaze, L.; Hocher, V.; Bogusz, D.; Smouni, A. Effect of lead on root growth. *Front. Plant Sci.* **2013**, *4*, 175.
(20) Jarvis, M. .; Leung, D. W. Chelated lead transport in *Pinus radiata*: An ultrastructural study. *Environ. Exp. Bot.* **2002**, *48* (1), 21−32.
(21) Inoue, H.; Fukuoka, D.; Tatai, Y.; Kamachi, H.; Hayatsu, M.; Ono, M.; Suzuki, S. Properties of lead deposits in cell walls of radish (*Raphanus sativus*) roots. *J. Plant Res.* **2013**, *126* (1), 51−61.
(22) Connolly, E. L.; Fett, J. P.; Guerinot, M. L. Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell* **2002**, *14* (6), 1347−1357.
(23) Clemens, S. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **2006**, *88* (11), 1707−1719.
(24) Pourrut, B.; Shahid, M.; Dumat, C.; Winterton, P.; Pinelli, E. Lead uptake, toxicity, and detoxification in plants. *Rev. Environ. Contam. Toxicol.* **2011**, *213*, 113−136.
(25) Gupta, D. K.; Huang, H. G.; Yang, X. E.; Razafindrabe, B. H. N.; Inouhe, M. The detoxification of lead in *Sedum alfredii* H. is not related to phytochelatins but to glutathione. *J. Hazard. Mater.* **2010**, *177* (1−3), 437−444.
(26) Maitani, T.; Kubota, H.; Sato, K.; Yamada, T. The composition of metals bound to class III metallothionein (phytochelatin and its dsglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. *Plant Physiol.* **1996**, *110* (4), 1145−1150.
(27) Gusmão, R.; Cavanillas, S.; Ariño, C.; Díaz-Cruz, J. M.; Esteban, M. Circular dichroism and voltammetry, assisted by multivariate curve resolution, and mass spectrometry of the competitive metal binding by phytochelatin PC5. *Anal. Chem.* **2010**, *82* (21), 9006−9013.
(28) Clemens, S. Evolution and function of phytochelatin synthases. *J. Plant Physiol.* **2006**, *163* (3), 319−332.
(29) Grill, E.; Löffler, S.; Winnacker, E.-L.; Zenk, M. H. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86* (18), 6838−6842.
(30) Andra, S. S.; Datta, R.; Sarkar, D.; Makris, K. C.; Mullens, C. P.; Sahi, S. V.; Bach, S. B. H. Synthesis of phytochelatins in vetiver grass upon lead exposure in the presence of phosphorus. *Plant Soil* **2010**, *326* (1−2), 171−185.
(31) Estrella-Gómez, N.; Mendoza-Cózatl, D.; Moreno-Sánchez, R.; González-Mendoza, D.; Zapata-Pérez, O.; Martínez-Hernández, A.; Santamaría, J. M. The Pb-hyperaccumulator aquatic fern *Salvinia minima* Baker, responds to Pb^{2+} by increasing phytochelatins via changes in SmPCS expression and in phytochelatin synthase activity. *Aquat. Toxicol.* **2009**, *91* (4), 320−328.
(32) Scheidegger, C.; Behra, R.; Sigg, L. Phytochelatin formation kinetics and toxic effects in the freshwater alga *Chlamydomonas reinhardtii* upon short- and long-term exposure to lead(II). *Aquat. Toxicol.* **2011**, *101* (2), 423−429.
(33) Howden, R.; Goldsbrough, P. B.; Andersen, C. R.; Cobbett, C. S. Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiol.* **1995**, *107* (4), 1059−1066.
(34) Ha, S.-B.; Smith, A. P.; Howden, R.; Dietrich, W. M.; Bugg, S.; O'Connell, M. J.; Goldsbrough, P. B.; Cobbett, C. S. Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. *Plant Cell* **1999**, *11* (6), 1153−1163.
(35) Tennstedt, P.; Peisker, D.; Böttcher, C.; Trampczynska, A.; Clemens, S. Phytochelatin synthesis is essential for the detoxification of excess zinc and contributes significantly to the accumulation of zinc. *Plant Physiol.* **2009**, *149* (2), 938−948.
(36) Kühnlenz, T.; Schmidt, H.; Uraguchi, S.; Clemens, S. *Arabidopsis thaliana* phytochelatin synthase 2 is constitutively active in vivo and can rescue the growth defect of the AtPCS1-deficient *cad1-3* mutant on Cd-contaminated soil. *J. Exp. Bot.* **2014**, DOI: 10.1093/jxb/eru195.
(37) Cao, S.; Chen, Z.; Liu, G.; Jiang, L.; Yuan, H.; Ren, G.; Bian, X.; Jian, H.; Ma, X. The Arabidopsis ethylene-insensitive 2 gene is required for lead resistance. *Plant Physiol. Biochem.* **2009**, *47* (4), 308−312.
(38) Kim, D.; Bovet, L.; Maeshima, M.; Martinoia, E.; Lee, Y. The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *Plant J.* **2007**, *50* (2), 207−218.
(39) Lee, M.; Lee, K.; Lee, J.; Noh, E. W.; Lee, Y. AtPDR12 contributes to lead resistance in Arabidopsis. *Plant Physiol.* **2005**, *138* (2), 827−836.
(40) Wheeler, D.; Edmeades, D. Effect of depth and lime or phosphorus-fertilizer applications on the soil solution chemistry of some New Zealand pastoral soils. *Soil Res.* **1995**, *33* (3), 461−476.
(41) Mehra, R. K.; Kodati, V. R.; Abdullah, R. Chain length-dependent Pb(II)-coordination in phytochelatins. *Biochem. Biophys. Res. Commun.* **1995**, *215* (2), 730−736.
(42) Piechalak, A.; Tomaszewska, B.; Baralkiewicz, D.; Malecka, A. Accumulation and detoxification of lead ions in legumes. *Phytochemistry* **2002**, *60* (2), 153−162.
(43) Zhang, Z.; Gao, X.; Qiu, B. Detection of phytochelatins in the hyperaccumulator *Sedum alfredii* exposed to cadmium and lead. *Phytochemistry* **2008**, *69* (4), 911−918.
(44) Andra, S. S.; Datta, R.; Sarkar, D.; Makris, K. C.; Mullens, C. P.; Sahi, S. V.; Bach, S. B. H. Induction of lead-binding phytochelatins in vetiver grass [*Vetiveria zizanioides* (L.)]. *J. Environ. Qual.* **2009**, *38* (3), 868−877.
(45) Zhu, Y.-G.; Chen, S.-B.; Yang, J.-C. Effects of soil amendments on lead uptake by two vegetable crops from a lead-contaminated soil from Anhui, China. *Environ. Int.* **2004**, *30* (3), 351−356.
(46) Xie, Z.; Wang, B.; Sun, Y.; Li, J. Field demonstration of reduction of lead availability in soil and cabbage (*Brassica chinensis* L.) contaminated by mining tailings using phosphorus fertilizers. *J. Zhejiang Univ., Sci., B* **2006**, *7* (1), 43−50.
(47) Grill, E.; Winnacker, E.-L.; Zenk, M. H. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proc. Natl. Acad. Sci. U. S. A* **1987**, *84* (2), 439−443.
(48) Vatamaniuk, O. K.; Mari, S.; Lu, Y.-P.; Rea, P. A. Mechanism of heavy metal ion activation of phytochelatin (PC) synthase blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. *J. Biol. Chem.* **2000**, *275* (40), 31451−31459.
(49) Oven, M.; Page, J. E.; Zenk, M. H.; Kutchan, T. M. Molecular characterization of the homo-phytochelatin synthase of soybean *Glycine max*: Relation to phytochelatin synthase. *J. Biol. Chem.* **2002**, *277* (7), 4747−4754.
(50) Kim, D.-Y.; Bovet, L.; Maeshima, M.; Martinoia, E.; Lee, Y. The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. *Plant J.* **2007**, *50* (2), 207−218.
(51) Samardakiewicz, S.; Krzeslowska, M.; Bilski, H.; Bartosiewicz, R.; Wozny, A. Is callose a barrier for lead ions entering *Lemna minor* L. root cells? *Protoplasma* **2012**, *249* (2), 347−351.
(52) Lummerzheim, M.; Sandroni, M.; Castresana, C.; De Oliveira, D.; Van Montagu, M.; Roby, D.; Timmerman, B. Comparative microscopic and enzymatic characterization of the leaf necrosis induced in *Arabidopsis thaliana* by lead nitrate and by *Xanthomonas campestris pv. campestris* after foliar spray. *Plant, Cell Environ.* **1995**, *18* (5), 499−509.