Accepted Manuscript

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PII: S0098-8472(19)30117-0
DOI: <https://doi.org/10.1016/j.envexpbot.2019.04.018>
Reference: EEB 3762

To appear in: *Environmental and Experimental Botany*

Received date: 28 January 2019
Revised date: 19 April 2019
Accepted date: 27 April 2019

Please cite this article as: Willick IR, Plaxton WC, Lolle SJ, Macfie SM, Transcriptional and Post-translational Upregulation of Phosphoenolpyruvate Carboxylase in *Arabidopsis thaliana* (L. Heynh) Under Cadmium Stress, *Environmental and Experimental Botany* (2019), <https://doi.org/10.1016/j.envexpbot.2019.04.018>

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Title:

Transcriptional and Post-translational Upregulation of Phosphoenolpyruvate Carboxylase in
Arabidopsis thaliana (L. Heynh) Under Cadmium Stress

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Highlights:

- PEPC responses to Cd, from mRNA transcripts to enzymatic products, were studied
- Transcript abundance for three isozymes of PEPC increased with Cd-treatment
- Phosphorylation and activity of PEPC increased in response to Cd
- Plants grown with Cd had higher concentrations of organic acids in the roots
- The isozyme AtPPC3 was important for reducing Cd stress

Abstract

Phosphoenolpyruvate carboxylase (PEPC) is a tightly controlled anaplerotic enzyme that replenishes organic acids that have been withdrawn from the tricarboxylic acid cycle. Organic acid anions are important chelators of toxic metals, including cadmium (Cd). To determine the Cd-stress responses of two isozymes of PEPC in Arabidopsis (AtPPC1 and AtPPC3), we compared wild type (Col-0) with two T-DNA insertional mutant lines (*atppc1* and *atppc3*). Plant mass decreased in response to Cd in all lines but *atppc3* was the most sensitive to Cd, even though its roots contained 30% lower concentrations of Cd. Treatment with 1 or 5 μM CdCl_2 resulted in up to 4-fold higher expression of genes that encode three isozymes of plant-type PEPCs and up to 2-fold higher expression of genes that encode two isozymes of PEPC kinase, especially in roots. The amount of PEPC and its phosphorylation status also increased by up to 40% with Cd treatment, as did enzyme activity (up to 2-fold). Relative abundances of transcripts in the mutant lines were as much as 50% lower than in wild type plants, even in control (no Cd) plants. The exception was *AtPPC2*, which had values equal to wild-type, likely indicating a compensatory response in lines lacking *AtPPC1* or *AtPPC3*. In general, concentrations of organic acid anions increased or stayed the same in response to Cd treatment in each plant line, although roots of mutant lines under control conditions had 25-50% less malate and citrate. Our results confirm the importance of AtPPC3 for the normal development of Arabidopsis, as well as the importance of AtPPC1 and AtPPC3 for tolerance to Cd.

Key words:

Arabidopsis thaliana, cadmium, low molecular weight organic acids, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase kinase, tricarboxylic acid cycle

Abbreviations:

107-kDa immunoreactive polypeptide (p107), 110-kDa immunoreactive polypeptide (p110), aluminum (Al), cadmium (Cd), Crassulacean acid metabolism (CAM), inductivity-coupled plasma atomic emission spectrometry (ICP-AES), phosphoenolpyruvate (PEP), phosphoenolpyruvate carboxylase (PEPC), phosphoenolpyruvate carboxylase kinase (PPCK), plant-type phosphoenolpyruvate carboxylase (PTPC), tricarboxylic acid cycle (TCA)

1. Introduction

Phosphoenolpyruvate (PEP) carboxylase (PEPC, EC 4.1.1.31) is an important cytosolic enzyme situated at a crucial branch point of plant carbohydrate metabolism. It catalyzes the irreversible β -carboxylation of PEP in the presence of HCO_3^- to yield oxaloacetate and inorganic phosphate (Pi). Crassulacean acid metabolism (CAM) and C_4 photosynthetic PEPCs have been widely studied owing to their central role in atmospheric CO_2 fixation (O'Leary et al., 2011b). PEPC also fulfills essential non-photosynthetic functions, particularly the anaplerotic replenishment of tricarboxylic acid (TCA) cycle intermediates consumed during biosynthesis and N-assimilation. Any developmental or metabolic process that requires these organic acids will benefit from increased anaplerotic carbon flux through the PEPC reaction.

Soil and water pollution by trace metals is a serious environmental concern. Among trace metal pollutants, cadmium (Cd) is one of the most phytotoxic. Although Cd occurs naturally, crop fertilization, landfills, and manufacturing contribute to its accumulation in agricultural soil (reviewed by Grant et al., 1998). Because of its high solubility in water, Cd is readily absorbed by roots (Sanità di Toppi and Gabbrielli, 1999). Even at low concentrations, Cd uptake by roots and transport to the vegetative and reproductive organs has a negative effect on plant growth and development. Intracellular cadmium (Cd) detoxification involves chelation of Cd^{2+} , the biologically active species of Cd, with phytochelatins in the cytosol and storage of Cd-citrate and Cd-malate complexes in the vacuole (Sanità di Toppi and Gabbrielli, 1999). Although the synthesis of phytochelatins is a well-known response of plants to Cd, in some species such as durum wheat (*Triticum durum* Desf.) there was no correlation between increased phytochelatin concentration and Cd translocation (Macfie et al., 2016). While Cd-phytochelatin complexes predominate in the phloem (Kutrowska and Szlag, 2014), Cd-citrate complexes are transported from root to shoot in the xylem, and Cd-citrate and Cd-malate complexes predominate in the vacuoles (Rauser, 1999).

Studies that assessed Cd-ligand binding using synchrotron x-ray absorption near-edge spectroscopy reported varying proportions of intracellular Cd bound to S-ligands, which represent phytochelatins and their thiol-containing precursors. For example, Akhter et al. (2014) reported that 75 to 88% of the Cd in barley (*Hordeum vulgare* L.) roots was bound to S-ligands whereas up to 50% of the Cd was bound O- and N-ligands in lettuce (*Lactuca sativa* L.) roots. The Cd in triochomes of *Arabidopsis halleri* ssp *gemmifera* was bound to O-ligands (Fukuda et al., 2008). The presence of Cd-O bonds could indicate chelation of Cd to the carboxylate groups of organic acid anions. Increased PEPC activity may, therefore, also alleviate Cd toxicity. Anaplerotic PEP carboxylation by PEPC not only results in malate and citrate accumulation but is also involved in *de novo* production of amino acids for glutathione synthesis, the precursor to phytochelatin (Nocito et al., 2008).

To achieve their diverse functions and complex regulation, vascular plant PEPCs belong to a small multi-gene family encoding several closely related plant-type PEPCs (PTPCs) as well as a distantly related, bacterial-type PEPC (O'Leary et al., 2011a). The genome of the model plant *Arabidopsis* (*Arabidopsis thaliana* L. Heynh) encodes three PTPC isozymes (AtPPC1, AtPPC2 and AtPPC3), and one distantly related bacterial-type PEPC (AtPPC4) (Fontaine et al., 2002; Sánchez and Cejudo 2003). The *PTPC* genes encode 100- to 110-kDa polypeptides that typically assemble as Class-1 PEPC tetramers, which are post-translationally controlled by several allosteric effectors in conjunction with reversible phosphorylation (activates) and mono-ubiquitination (inhibits) (Uhrig et al., 2008; Gregory et al., 2009; O'Leary et al., 2011a). By contrast, bacterial-type *PEPC* genes encode 116–118 kDa polypeptides that exhibit low sequence identity with PTPCs and are more closely related to prokaryotic PEPCs than to PTPCs (O'Leary et al., 2011a, 2011b). Studies of PEPC from green algae, developing castor oil (*Ricinus communis* L.) seeds, and pollen of *Arabidopsis* and lily (*Lilium longiflorum* cv. Hinomoto) led to the discovery of high- M_r Class-2 PEPC hetero-octameric complexes composed of tightly associated PTPC and bacterial-type PEPC subunits (Rioval et al., 2001;

Igawa et al., 2010; O'Leary et al., 2011a). The Class-2 PEPC complexes are largely desensitized to allosteric effectors compared to Class-1 PEPCs. Although bacterial-type PEPC transcripts have been detected in *Arabidopsis* rosette leaves and roots, expression of bacterial-type PEPC polypeptides or Class-2 PEPC complexes appears to be restricted to developing pollen (Sánchez et al., 2006; Igawa et al., 2010).

A calcium-independent PTPC protein kinase (PPCK, EC 2.7.11.1) catalyzes the phosphorylation of PTPCs at their conserved N-terminal seryl residue (Vidal and Chollet, 1997). In *Arabidopsis*, there are two PPCK isozymes: AtPPCK1 and AtPPCK2 (Fontaine et al., 2002). Phosphorylation enhances allosteric activation of Class-1 PEPCs by hexose-phosphates while reducing their inhibition by L-malate and L-aspartate (O'Leary et al., 2011b). Due to the importance of phosphorylation as a regulatory mechanism of photosynthetic and non-photosynthetic Class-1 PEPCs, PPCK-mediated phosphorylation of Class-1 PEPC continues to provide one of the best-understood paradigms of regulatory enzyme phosphorylation in the plant kingdom.

While many have measured changes in PEPC transcripts and activity in response to abiotic stress, only a few have investigated the relative contributions of individual PEPC or PPCK isozymes, and/or the adaptive role that *in vivo* PEPC phosphorylation plays in stress tolerance. Under control conditions, *AtPPC1* and *AtPPC3* transcript abundances were 3- and 50-fold higher in *Arabidopsis* roots compared to shoots, respectively, whereas *AtPPC2* was preferentially expressed in shoots (Feria et al., 2016). The individual isozyme responses to stress vary. For example, Gregory et al. (2009) reported that Pi deprivation of *Arabidopsis* seedlings or suspension cells increased *AtPPC1*, *AtPPC2* and *AtPPC3*, as well as *AtPPCK1* and *AtPPCK2* transcription. This was paralleled by phosphorylation of AtPPC1 into its more active form (Gregory et al., 2009) and increased anaplerotic PEP carboxylation *in vivo* (Masakapalli et al., 2014). In iron-deficient *Arabidopsis*, *AtPPCK1* expression increased in root tissues whereas *AtPPCK2* did not (Lan et al., 2012). The genes encoding isozymes of PEPC also respond differentially to salinity

and drought. Sánchez et al. (2006) determined that *AtPPC2* is a 'housekeeping' gene that maintains basal metabolism, even under stress conditions, and transcripts for each of *AtPPC1* and *AtPPC3* increased in response to salt but not drought. Feria et al. (2016) reported that *AtPPC3* knockout plants were more sensitive to both phosphate deficiency and salt stress. Because *AtPPC2* seems to be less important for stress tolerance, *AtPPC1* and *AtPPC3* were the primary focus of our study.

Given the importance of organic acid anions to metal detoxification in general and Cd detoxification in particular, as well as the importance of PEPC in the synthesis of organic acid anions and phytochelatins, this study was designed to test the hypothesis that exposure to Cd would increase *AtPPC* gene expression and PEPC activity. To gain a complete picture (i.e., gene expression through enzyme activity to TCA cycle product) of PEPC-related responses to Cd, we compared responses of *Arabidopsis* Col-0 (wild-type) with T-DNA insertional mutant lines for *AtPPC1* or *AtPPC3*. Plant shoots and roots were analyzed for: (i) changes in biomass and rosette diameter in response to Cd treatment; (ii) relative transcript abundance for each PTPC and PPCK isozyme; (iii) activity, and relative abundance and phosphorylation status of PEPC; (iv) PPCK activity; and (v) concentrations of organic acid anions. This work contributes to our understanding of the role of selected PEPC-related enzymes in plant growth under Cd-stressed conditions.

2. Materials and methods

2.1 Plant growth

All *Arabidopsis thaliana* plants used in this study were from the Columbia (Col-0) background. Seeds of the T-DNA insertional mutant SALK lines, *atppc1* (SALK_070605C) and *atppc3* (SALK_031519C), were obtained from the Arabidopsis Biological Resource Center. Mutant lines were analyzed using PCR (see Supplementary material Table S1 for the list of primers) to verify T-DNA insertion within the *atppc1* or *atppc3* alleles in the respective mutant lines (Supplementary material Fig. S1) prior to experimentation.

Reciprocal crosses between *atppc1* and *atppc3* mutant lines were done manually. Plants homozygous for *atppc1* and heterozygous for *atppc3* or homozygous for *atppc3* and heterozygous for *atppc1* were allowed to self-fertilize and progeny genotyped by PCR using allele-specific primers (Table S1) to identify double mutants. In excess of 400 progeny were genotyped.

Seeds were surface-sterilized and stored at 4°C in the dark for 3 days (d) to synchronize germination as described by Hétu et al. (2005). To measure rosette diameter, chlorophyll fluorescence, and chlorophyll content, seeds were transferred directly to sterile Petri plates containing 20 mL of half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 1% (w/v) sucrose, 0.8% (w/v) agar and selected concentrations of CdCl₂; pH was adjusted to 5.8. In agar and hydroponic media (below), Cd²⁺ was readily available for plant uptake and no Cd-induced P-deficiency occurred (Supplementary material Fig. S2).

To quantify biomass, concentrations of Cd and organic acids in roots and shoots, as well as the investigations of the transcript abundance, activity and phosphorylation of PEPC and PPCK, and production of organic acids, seeds were germinated on nutrient agar and then transferred to liquid MS medium. Before seed transfer, sterile 2.5 cm² pieces of fiberglass screen (Easy Screen, RCR International Inc., Quebec) were placed on 20 mL of half-strength MS medium (described above) in sterile Petri plates. Batches of 15 seeds were pipetted onto mesh screens, Petri plates were sealed with Parafilm, then placed in a growth chamber with a 16:8 h light: dark cycle and maintained at 60 % RH and 22°C. Light intensity at the seedling level was determined to be 185 ± 6 μmol m⁻² s⁻¹ using a Li-250 light meter (LiCor Inc., Nebraska).

After 7 d, mesh screens and seedlings were transferred into sterile glass jars containing 20 mL half-strength MS medium (with 1 % (w/v) sucrose, CdCl₂ treatments, pH 5.8). The jars' openings were sealed with double-layered aluminum foil to avoid contamination. Jars were returned to the growth chamber and placed on an orbital shaker (70 rpm). Light intensity at the seedling level was 113 ± 6 μmol m⁻² s⁻¹ due to light attenuation through the glass and presence of the foil lid. Plants were transferred to fresh 20 mL media every 7 d. Petri plates and jars were

rotated daily to minimize position-dependent variation in growth chamber conditions. At 21 d in the growth chamber, plants were harvested.

2.2 *Biomass and rosette diameter determination*

For biomass measurements, Arabidopsis were grown hydroponically as described above for 21 d and the number of seedlings per plate was recorded. The experimental treatments included 0, 0.1, 1.0, 5.0, 7.5, or 10 μM CdCl_2 in the nutrient agar, with four replicates per treatment. After 21 d, fresh weights of roots and shoots were recorded, each tissue was placed in pre-dried, pre-weighed Al foil envelopes, then oven-dried at 60°C to constant weight prior to recording root and shoot dry weight.

To quantify rosette diameter, Arabidopsis were grown on agar plates as described above with CdCl_2 (0, 0.1, 1.0, 5.0, 7.5, or 10 μM) for a total of 14 d. Five seeds were pipetted onto each of four replicate plates, no less than 2 cm apart and away from the plate's edge. Each plate was photographed and the maximum rosette diameter for each plant was measured using ImageJ software version 1.44. To calibrate the software, a subset of rosette diameters were also measured using digital calipers.

2.3 *Determination of maximum photochemical efficiency of photosystem II and shoot chlorophyll content*

Seedlings were grown on agar plates as described above, with four replicates of three CdCl_2 treatments (0, 1, 5 μM) for 14 d. Following dark-adaptation for 1 h at room temperature, plant shoots were exposed to a single short (800 ms) pulse of saturating blue light ($\lambda = 470$ nm, 6000 $\mu\text{mol m}^{-2}\text{s}^{-1}$) from the PAM photodiode (IMAGE-L, Heinz Walz, Germany). Fluorescence measurements (variable fluorescence, F_v , and maximum fluorescence, F_m) were captured using an Imaging PAM Chlorophyll Fluorometer (Heinz Walz, Germany) to calculate the maximum photochemical efficiency of PSII (F_v/F_m).

A 0.1 g sub-sample of shoot tissue was frozen in liquid nitrogen and ground to powder in a chilled mortar. Chlorophyll was isolated as described by Pocock et al. (2004). A 200 μ L aliquot was added to a 96 well plate. Absorbance was measured at 664, 647, and 750 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, California). Chlorophyll content and the chlorophyll a:b ratio was calculated using equations as described by Porra et al. (1989).

2.4 Cadmium extraction and quantification

For the remaining experiments, Arabidopsis were grown hydroponically as described above for 21 d in liquid MS medium containing sucrose, 0, 1 or 5 μ M CdCl₂, with 3 replicates per treatment. At harvest, plants were rinsed in RO (reverse osmosis) water, roots and shoots were separated, then oven-dried to constant weight. Dried tissue was ground into a fine powder and then weighed (0.050 g shoots, 0.025 g roots) into an acid-washed test tube. The concentration of Cd in roots and shoots was determined by hot acid digestion using a method modified from the United States Environmental Protection Agency test method SW-846 (US EPA 2011). A standard reference material from the National Institute of Standards and Technology (NIST #1573a, trace elements in tomato leaves) and reagent blanks were included in the analysis. After digestion and filtration, the samples were analyzed for Cd content by inductivity-coupled plasma atomic emission spectrometry (ICP-AES).

2.5 RNA extraction and quantification

Total RNA was extracted as using Trizol Reagent as described by the manufacturer's instructions (Invitrogen, California). Absorbance was measured at 260 and 280 nm to determine RNA concentration and subsamples were run on an agarose gel to assess RNA integrity. Samples were treated with DNase I (Invitrogen, California) and RNA (1 μ g) was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Massachusetts). Quantitative PCR was performed

with the Bio-Rad CFX96 Real Time PCR detection system (Bio-Rad, California) and PerfeCTa SYBR Green FastMix (Quanta Biosciences, Massachusetts) using the following cycling program. One cycle (95°C for 2 min), 40 cycles (95°C for 10 s, 53°C for 15 s, 72°C for 10 s followed by fluorescence measurements every 10 s per 0.5°C increment increase from 68 to 72°C). Gene-specific target primers (Table A1) were used to amplify *AtPPC1*, *AtPPC2*, *AtPPC3* (Sánchez and Cejudo, 2003), *AtPPCK1* and *AtPPCK2* (Fontaine et al., 2002). Ubiquitin 10 (*AtUBQ10*) (Divi et al., 2010) was used as the reference gene. Relative transcript abundance was calculated using the delta CT method (Rao et al., 2013).

2.6 Protein extraction and quantification

Roots and shoots were harvested, frozen with liquid nitrogen and then stored at -80°C. Total protein used for PEPC measurements was extracted as described by Gregory et al. (2009), while total protein used for PPCK measurements was extracted and desalted using MicroSpin G-50 columns (GE-Healthcare), as described by Murmu and Plaxton (2007). Protein samples were quantified using a protein assay (500-0006, Bio-Rad) and standardized with a gamma globulin (Bio-Rad) dilution curve. All assays were linear with respect to time and concentration of enzyme assayed.

2.7 PEPC activity assay

The activity of PEPC was assayed at 24°C as described by Gregory et al. (2009) using an aliquot from the PEPC total protein extracts. Absorbance was immediately measured at 340 nm using a Spectramax M2 Microplate Reader (Molecular Devices, Sunnyvale, California). Samples were run in duplicate and all enzyme assays were linear with respect to time and concentration of enzyme assayed. Three replicates were included for each experimental group. One unit of activity was defined as the amount of PEPC resulting in the production of 1 μ mol of oxaloacetate per min.

2.8 Semi-quantification of PEPC amount and phosphorylation status using immunoblotting

Homogeneous, *in vivo* phosphorylated AtPPC1 purified from Pi-starved Arabidopsis suspension cells (Gregory et al., 2009) was used as a positive control for PEPC on immunoblots. Protein extracts were heated along with a PEPC control sample for 5 min at 90°C and then fractionated using SDS-PAGE as described by Tsang et al. (1983) on an equal protein basis (20 µg per lane). Proteins were blotted onto a nitrocellulose membrane (Bio-Rad) and then blocked for 1 h at room temperature with 5% (w/v) skim milk Tris-buffered saline [20 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween20, pH 7.6]. Membranes were washed three times with tris-buffered saline for 5 min each. Membranes were then incubated for 1 h in a 1:2000 dilution of rabbit anti-[castor seed Class-1 PEPC (RcPPC3)]-immune serum (anti-p107) or a polyclonal antibody raised against a synthetic phosphopeptide matching the conserved N-terminal Ser11 phosphorylation domain of the castor seed Class-1 PEPC (anti-pSer11) (Tripodi et al., 2005). For all anti-pSer11 immunoblots, the corresponding deP-peptide (10 µg mL⁻¹) was used to block any nonspecific antibodies raised against non-phosphorylated portions of the sequence. The blot was washed for 1 h and incubated for 1 h with goat anti-rabbit peroxidase-linked secondary antibody (G-7641, Sigma). Following one final wash period of 1 h, immunodetection was performed using chemi-luminescence according to the manufacturer's instructions (ECL, Amersham-Pharmacia Biotech). Band intensity was quantified using Image J software and relative phosphorylation was quantified by taking the ratio of pSer11 and p107 band intensities.

2.9 PPCK activity assay

The PPCK activity was assayed as described by Murmu and Plaxton (2007) with the following modifications. A 50 µg sample of PPCK protein extract and 10 µg of purified PEPC were dephosphorylated in dephosphorylation buffer [50 mM Tris-HCl, 5 mM MgCl₂, 1 mM DTT, 20% (v/v) glycerol, 5 mU mL⁻¹ of the catalytic subunit of bovine protein phosphatase type-2A (PP2A)] and incubated at 30°C for 30 min. The PEPC control was *in vivo* phosphorylated AtPPC1 purified

from phosphate-starved *Arabidopsis* suspension cells (Gregory et al., 2009). Following dephosphorylation, samples were incubated with the PPCK assay mix. Addition of ATP initiated the reaction. Samples were incubated at 30°C for 15 min, heated at 90°C for 5 min and subjected to SDS-PAGE and immunoblotting using anti-pSer11 or anti-p107. A preliminary PPCK time course assay was conducted as outlined above, with samples removed at 0, 5, 10, 15, 20, 25, and 30 min after the addition of PPCK assay mix. Band intensity was measured using image J software. Relative rates of phosphorylation were calculated by taking the ratio of anti-pSer11 to anti-p107 immunoreactive polypeptides, as measured on 3 replicate immunoblots.

2.10 Organic acid extraction and quantification

Organic acids were extracted as described by Xu et al. (2006) with the following modifications. Root and shoots were ground to a fine powder with liquid nitrogen in a chilled mortar. A subsample was weighed (0.5 g shoots, 0.25 g roots) into a clean acid-washed test tube with chilled 0.5 M HCl (3 mL shoots, 1.5 mL roots). Test tubes were stoppered with an acid-washed marble and transferred to a hot water bath set at 80°C for 20 min. Every 5 min the samples were agitated. Samples were removed from the hot water bath, cooled to room temperature, transferred to 15 mL Falcon tubes and then diluted to a total volume of 10 mL for shoot samples or 5 mL for root samples. Samples were centrifuged at 2500 × g for 15 min and the supernatant was collected and filtered (0.45 µm) into a clean, sterile 15 mL Falcon tube then stored at 4°C prior to analysis.

Separation and quantification of organic acids by RP-HPLC (reverse phase high performance liquid chromatography) as described by Xu et al. (2006) using an Agilent Infinity 1260 HPLC (Binary Pump, Agilent Technologies, USA). A 20 µL aliquot of sample was injected onto a Prevail Organic Acid HPLC column (150 × 4.6 mm 5 µM, Alltech, Mandel Scientific Company, Guelph, Ontario) with a mobile phase of 25 mM KH₂PO₄, adjusted to pH 2.5 with phosphoric acid, at a flow rate of 1.0 mL min⁻¹. Organic acid anions were detected at 210 nm with a UV detector. Standards for oxalate, malate, succinate, citrate and fumarate (Sigma) were made in RO water

and calibration curves were generated for each. The identities of the organic acids were confirmed by spiking samples with organic acid standards. To determine the percentage of organic acids recovered during the digestion process, a mixture of standard acids was processed with the samples. A mixture of standard acids (and a duplicate) verified stability of running conditions before each batch of samples. After every 20 samples an HCl and RO water filtration blank were included and after every 15 samples, a mixture of standard acids and a sample duplicate were included to confirm column integrity and repeatability of the separation and quantification of the chromatographic peaks.

2.11 Statistical analysis

Normality and homogeneity of variance was confirmed prior to analysis and in instances where non-normal distributions were identified (relative transcript abundance and organic acid concentrations), data required log transformation to achieve normal distribution. Analyses of tissue Cd concentrations, relative transcript abundance, PEPC and PPCK activity, relative PEPC phosphorylation and organic acid concentrations was performed with the SigmaPlot 12 (Systat Software Inc., Chicago, IL, USA) three-way ANOVA procedure. Analysis of mean rosette diameter, chlorophyll a:b ratios, total chlorophyll content and F_v/F_m , was performed with the two-way ANOVA procedure. Tukey's post hoc test was performed to detect CdCl_2 , line and/or organ-specific effects.

3. Results

3.1 Effect of Cd treatment on plant phenotype

Root and shoot biomass of all plant lines decreased as the concentration of CdCl_2 in the growth medium increased (Fig. 1). Across all CdCl_2 treatments, *atppc3* plants were consistently the smallest. Similar patterns were seen for rosette diameter (Supplementary material Fig. S3). To assess relative tolerance of each plant line to Cd, the total biomass of plants (shoots plus roots)

grown with 10 μM Cd was compared to the total plant biomass under control conditions. Relative to their respective controls, Col-0 plants from the 10 μM Cd treatment were 40% smaller, *atppc1* plants were 67% smaller and *atppc3* plants were 78% smaller, leading to the conclusion that, of the isozymes tested, AtPPC3 was the most important to the plant for resistance against Cd toxicity. Double mutant plants harbouring T-DNA insertions in both *atppc1* and *atppc3* could not be recovered either as adults or at seedling stages. Further investigation revealed evidence of seed abortion among segregating plants suggesting that double mutants are embryo lethal (see Supplementary material Fig. S4).

Figure 1. Impact of Cd treatment on shoot versus root biomass of 28-d old, hydroponically grown wild-type *Arabidopsis* (Col-0), *atppc1* and *atppc3*. Shoot (top) and root (bottom) dry weight per plant; error bars represent standard error.

When investigating differential Cd accumulation patterns as well as molecular and biochemical responses to Cd, it is important that the plants were not exposed to highly toxic concentrations of Cd; the plants should be under only mild Cd stress. The seedlings with visible symptoms of Cd-induced stress (leaf chlorosis) were grown with 7.5 or 10 μM CdCl_2 . Taken together with the results for biomass, subsequent experiments were performed with 0, 1, or 5 μM CdCl_2 .

In all plant lines, the chlorophyll a:b ratio was unaffected by 1 or 5 μM CdCl_2 (2-way ANOVA, $P \geq 0.97$, $n = 8$). In *Arabidopsis* grown with 0, 1 and 5 μM CdCl_2 , the ratio of chlorophyll a:b was 2.24 ± 0.19 ($n = 72$; all plants pooled). However, total chlorophyll (a + b) was 1.2 to 1.6-fold lower in *Arabidopsis* grown in 5 μM CdCl_2 compared to *Arabidopsis* grown in the absence of CdCl_2 (2-way ANOVA, $P \leq 0.05$, $n = 8$). The maximum PS II photochemical efficiency (F_v/F_m) did not vary among plants from 0, 1 and 5 μM CdCl_2 treatments (2-way ANOVA, $P = 0.96$, $n = 8$) and, overall, $F_v/F_m = 0.788 \pm 0.015$ ($n = 72$; all plants pooled).

The concentrations of Cd in the tissues increased as the concentrations of CdCl_2 in the growth medium increased (Fig. 2). For each CdCl_2 treatment, Cd concentrations in roots were 3-fold-higher than in the shoots. *Arabidopsis* grown in 5 μM CdCl_2 accumulated 5-fold higher concentrations of Cd in the shoots and 4-fold higher concentrations in the roots compared to those grown in 1 μM CdCl_2 . Wild-type and *atppc1* grown in 5 μM CdCl_2 accumulated 1.4-fold higher concentrations of Cd in the roots compared to *atppc3*. Although roots of *atppc3* plants contained about 45% lower concentrations of Cd, the biomass of Cd-treated plants was proportionately smaller. This also indicates that the AtPPC3 isozyme is especially important for Cd tolerance.

3.2 PTPC and PPCK transcriptional expression

Disruption of the genes in the T-DNA mutant lines were verified; the corresponding transcripts were below the detection limit of qPCR (Fig. 3A, 3C). In general, the relative abundance of *AtPPC1*, *AtPPC2*, *AtPPC3* transcripts (Fig. 3) as well as *AtPPCK1* and *AtPPCK2* transcripts (Fig.

4) increased in response to Cd. Levels of these transcripts in Arabidopsis grown with 5 μM CdCl_2 were equal to or higher than those grown with 1 μM CdCl_2 .

With the exception of *AtPPC2* (Fig. 3B), transcripts were consistently more abundant in roots than in shoots. In wild-type plants, the difference between roots and shoots was particularly large for *AtPPC1* (Fig. 3A) and *AtPPC3* (Fig. 3C), which were 8- to 20-fold higher in roots. In the roots of wild-type treated with 5 μM CdCl_2 , there was a 4- and 2-fold increase in *AtPPC1* (Fig. 3A) and *AtPPC3* (Fig. 3C) transcripts, respectively, compared to non-Cd treated control plants.

When comparing the mutant lines relative to wild-type from the 5 μM CdCl_2 treatment, roots of *atppc3* accumulated 50% less *AtPPC1* (Fig. 3A) but 2-fold more *AtPPC2* (Fig. 3B). While abundance of *AtPPC1* in shoots was 30% lower in *atppc3* compared to wild-type (Fig. 3A), levels of *AtPPC2* were the same (Fig. 3B). For *atppc1*, transcript abundance for *AtPPC2* was equal to that of wild-type in shoots but 25% higher than wild-type in roots (Fig. 3B). Levels of *AtPPC3* in *atppc1* were equal to wild-type in shoots and 75% lower in roots (Fig 3C).

Overall, the results for wild-type *AtPPCK1* and *AtPPCK2* (Fig. 4) were similar to those of *AtPPC1* and *AtPPC3*: relative transcript abundance decreased with increasing Cd treatment, transcript abundance was higher in roots than in shoots, and transcript abundances in the roots of mutant lines were up to 50% lower than in wild-type.

Figure 2. Concentrations of Cd in shoots (top) and roots (bottom) of wild-type Arabidopsis (Col-0), *atppc1* and *atppc3* grown with 0, 1 or 5 μM CdCl₂. Vertical error bars represent standard error. In some treatments, Cd concentration was below the detection limit (bdl). Concentrations with different letters are significantly different (3-way ANOVA with Tukey's post hoc test, $P \leq 0.05$, $n = 3$).

Figure 3. Relative *AtPPC1* – *AtPPC3* transcript abundance in wild-type Arabidopsis (Col-0), *atppc1* and *atppc3*. Levels of (A) *AtPPC1*, (B) *AtPPC2*, and (C) *AtPPC3* mRNA in Arabidopsis grown with 0, 1, or 5 μ M CdCl₂ were measured using quantitative RT-PCR and gene-specific primers (Table A1). *UBQ10* was the reference gene. Vertical error bars represent standard error. Relative mRNA levels with different letters are significantly different (3-way ANOVA with Tukey post hoc test, $P \leq 0.05$, $n = 3$).

Figure 4. Relative *AtPPCK1* and *AtPPCK2* transcript abundance in wild-type Arabidopsis (Col-0), *atppc1* and *atppc3*. Levels of (A) *AtPPCK1* and (B) *AtPPCK2* mRNA in Arabidopsis grown with 0, 1, or 5 μ M CdCl_2 were measured using primers specific for *AtPPCK1* and *AtPPCK2* (Table A1). *UBQ10* was the reference gene. Vertical error bars represent standard error. Relative mRNA levels with different letters are significantly different (3-way ANOVA with Tukey post hoc test, $P \leq 0.05$, $n = 3$).

3.3 PEPC abundance, phosphorylation state, and specific activity

Immunoblots were used to determine the relative amount (Fig. 5A), phosphorylation status (Fig. 5B) and the activity of PTPC (Fig. 5C), as well as the activity of PPCK (Fig. 6A). Antibodies specific to isozymes of PTPC and PPCK were not available so the results represent relative phosphorylation of all PTPC isozymes and total PPCK activity.

An immunoreactive 107-kDa polypeptide (p107, Fig. 5D) co-migrated with the p107 subunit of homogeneous, phosphorylated AtPPC1 purified from Pi-starved Arabidopsis suspension cells (Gregory et al., 2009). For wild-type, relative p107 band intensity was about 20% higher in shoots compared to roots, and increased with increasing Cd treatment in roots and shoots (Fig. 5A). In *atppc1* and *atppc3*, relative band p107 intensity was up to 50% lower than in wild-type, in control and with Cd treatment. A second immunoreactive 110-kDa polypeptide (p110) was detected to a greater degree in the wild-type compared to *atppc1* or *atppc3* on immunoblots of shoot extracts (Fig. 5D), which is indicative of p107 monoubiquitination (O'Leary et al., 2011a). The relative amount of p110 in shoot extracts did not vary with Cd treatment (1-way ANOVA, $P = 0.82$, $n = 4$).

Immunoblotting with anti-[castor bean PTPC pSer11 phosphosite specific] antibodies (anti-pSer11) (Tripodi et al., 2005) indicated the p107 subunit of PTPCs was phosphorylated at their conserved N-terminal seryl phosphorylation sites in roots and shoots at all concentrations of CdCl_2 (Fig. 5B, D). In wild-type, relative p107 phosphorylation was 1.9-fold higher in shoots compared to roots and increased 1.4-fold in response to $5 \mu\text{M}$ CdCl_2 . Loss of *AtPPC1* function resulted in a 50% decrease in relative p107 phosphorylation in shoots and roots. In contrast, a loss of *AtPPC3* function resulted in a 75% reduction in shoot relative p107 phosphorylation and no change in roots. The specific activity of PEPC extracted from wild-type roots and shoots increased 1.5- to 2-fold with increasing CdCl_2 in the growth media (Fig. 5C). Among all plant lines, there was a 2-fold greater PEPC activity in roots relative to shoots. Plants grown with $5 \mu\text{M}$ CdCl_2 with a loss of *AtPPC1* or *AtPPC3* function had a 1.3 to 1.5-fold reduction in PEPC activity.

Figure 5. Influence of Cd stress on PEPC activity, amount, and phosphorylation status. (A) The p107 band intensity and (B) p107 phosphorylation status and (C) PEPC specific activity in the shoots (top) and roots (bottom) of wild-type (Col-0), *atppc1* or *atppc3* exposed to 0, 1, or 5 μ M CdCl₂. (E) Clarified extracts from shoots (0S, 1S, 5S) or roots (0R, 1R, 5R) were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane. Immunoblots were probed with anti-p107 or an anti-pSer11 antibody raised against the conserved N-terminal Ser11 phosphorylation domain of a castor plant-type PEPC isozyme (Tripodi et al., 2005). AtPPC represents phosphorylated AtPPC1 purified from Pi-starved Arabidopsis suspension cells (Gregory et al., 2009). Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (2-way ANOVA with Tukey post hoc test, $P \leq 0.05$, $n = 3$).

In wild-type, relative PPCK activity did not vary with CdCl₂ treatment in the shoot or root, although values for roots were 2-fold higher compared to shoots (Fig 6). In comparison with the wild-type plants, *atppc1* and *atppc3* had up to 50% lower shoot PPCK activity. In Cd-treated roots, activity was the same as wild-type for *atppc1* but up to 40% lower than wild-type for *atppc3*.

Figure 6. Influence of Cd stress on PPCK activity. (A) The relative PPCK activity in the shoots (top) and roots (bottom) of wild-type (Col-0), *atppc1* and *atppc3* exposed to 0, 1, or 5 μ M CdCl₂. (E) Dephosphorylated AtPPC1 from Pi-starved Arabidopsis suspension cells was incubated in a reaction mixture containing MC-LR and PP2A-treated, desalted and clarified shoot and root extracts. Clarified extracts from shoots (0S, 1S, 5S) or roots (0R, 1R, 5R) were subjected to SDS-PAGE and electroblotted onto a nitrocellulose membrane. Immunoblots were probed with anti-pSer11 antibody raised against the conserved N-terminal Ser11 phosphorylation domain of a castor plant-type PEPC isozyme (Tripodi et al., 2005). Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test, $P \leq 0.05$, $n = 3$).

3.4 Organic acid anions

Apart from malate, higher concentrations of organic acid anions accumulated in the shoots than in the roots, and oxalate was the most abundant (Fig. 7). Root and shoot concentrations of fumarate (Fig. 7A) and malate (Fig. 7B) were low (less than 0.4 mg g^{-1} of fresh weight) or below detectable limits. Concentrations of malate in shoots dropped by 30% in *atppc1* compared to the other two lines. In roots, concentrations of malate were 25% lower than wild-type for *atppc1* and 50% lower for *atppc3*.

With only a few exceptions, concentrations of the other organic acid anions in shoots did not vary with Cd treatment; the largest effects were found in roots (Fig. 7C-E). In wild-type roots, concentrations of oxalate, citrate and succinate increased 1.2, 1.4 and 2.4-fold, respectively, in response to $5 \text{ } \mu\text{M}$ CdCl_2 (Fig. 7E, D and C, respectively). In *atppc1* roots, oxalate increased by 20% in response to Cd but the other anions did not vary with treatment. In *atppc3* roots, concentrations of succinate and citrate doubled in response to $5 \text{ } \mu\text{M}$ Cd. In all lines, total concentration of organic acid anions was 2-fold greater in shoots than in roots, and either stayed the same or increased in response to Cd (Fig. 7F). It should be noted that organic acid anions were measured in bulk organs (shoots and roots) and that there might have been differences in their concentrations among subcellular compartments (e.g., cytosol versus vacuole).

Figure 7. Impact of Cd-stress on levels of various organic acid anions in wild-type Arabidopsis (Col-0), *atppc1* and *atppc3* shoots and roots. Concentration of (A) fumarate, (B) malate, (C) succinate, (D) citrate, (E) oxalate, and (F) their combined total shoots (top) and roots (bottom) from Arabidopsis grown with 0, 1, or 5 μM CdCl_2 . Vertical error bars represent standard error. Concentrations with different letters are significantly different (3-way ANOVA with Tukey post hoc test, $P \leq 0.05$, $n = 3$).

4. Discussion

4.1 Exposure to Cd reduced plant growth

Accumulation of Cd in the roots and shoots in combination with the reduced growth and total chlorophyll content of seedlings grown in the 1 and 5 μM CdCl_2 treatments confirms that Arabidopsis was under Cd stress in our experiment. Even so, Cd did not affect the chlorophyll a:b ratio or the maximum photochemical efficiency of PS II (F_v/F_m), indicating that the stress was not severe.

Walters and Horton (1994) reported chlorophyll a:b ratios of 3.5 and 2.8 for Arabidopsis grown under control conditions at 400 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. The low chlorophyll a:b ratio in our study (2.2) was likely a result of the light intensity in the growth chamber; it is similar to that of Aluru et al. (2001) who cultivated Arabidopsis at a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The low concentrations of Cd in our experiment (1 and 5 μM CdCl_2) were not expected to affect F_v/F_m . Others have studied the effect of Cd on photochemical efficiency in Arabidopsis, notably Martínez-Peñalver et al. (2012) who reported F_v/F_m initially decreased in plants grown with either 50 or 100 μM CdCl_2 but returned to control values (0.8) after 24 h. However, when Maksymiec et al. (2007) grew Arabidopsis in 100 μM CdCl_2 for 12 d, F_v/F_m decreased to 0.7.

The observed differences in dry weight and rosette diameter between wild-type, *atppc1* and *atppc3* are likely a result of a plant's inability to produce organic acids at a sufficient rate if one of the isozymes is missing. Several processes consume organic acids including excretion of organic acids that facilitate nutrient (Pi, Fe, Zn) uptake or Al^{3+} detoxification (Salt et al., 1999; O'Leary et al., 2011b; Kochian et al., 2015) and production of amino acids needed for protein, glutathione, and phytochelatin synthesis (Nocito et al., 2008). The consistently small relative size of the *atppc3* mutant indicates that AtPPC3 is important for routine anaplerotic replenishment of TCA cycle intermediates withdrawn for biosynthesis; although the other isozymes were able to maintain some anaplerotic C-flux.

Roots of *atppc1* and *atppc3* seedlings were more sensitive to Cd than were those of the wild-type. Since the roots accumulated 2 to 3 times more Cd than did shoots, this result is not surprising. However, the increased sensitivity of *atppc1* and *atppc3* to Cd, indicates *AtPPC3* and *AtPPC1* function are both associated with the chelation and/or sequestration of Cd, or in the repair of Cd-induced damage. In our study, *atppc3* was more sensitive than *atppc1* to Cd stress.

Plants also produce organic acids in response to Pi-deficiency; the synthesis and *in vivo* phosphorylation of *AtPPC1* increased in Pi-deficient Arabidopsis (Gregory et al., 2009). The similar responses of *AtPPC1* to Cd and Pi stress are consistent with the hypothesis that *AtPPC1* plays an important role in general metabolic adaptations to nutrient/metal stress. It is also possible that intracellular Cd formed complexes with P in the vacuoles. If this was the case, then the sensitivity of the *atppc1* mutant to Cd treatment might also be related to the importance of *AtPPC1* in mediating P-deficiency.

4.2 Response of the PEPC pathway to Cd

From *AtPPC1-AtPPC3* transcript abundance through to *in vivo* phosphorylation status and PEPC activity, Cd stimulated each step in the pathway. Under control conditions, *AtPPC1* and *AtPPC3* expression was higher in roots compared to shoots, whereas *AtPPC2* expression was higher in shoots compared to roots, which corroborates the results of Gregory et al. (2009). When grown with CdCl_2 , *AtPPC1-AtPPC3* were transcriptionally induced. In contrast, when Arabidopsis experienced other abiotic stressors not all the genes encoding these PTPC isozymes responded. For example, *AtPPC2* transcripts did not increase in response to Fe-deficiency (Lan et al., 2012), whereas *AtPPC3* was downregulated in plants grown under drought or saline conditions (Sánchez et al., 2006). In contrast, Feria et al. (2016) reported a significant increase in root *AtPPC3* and *AtPPCK2* transcripts when Arabidopsis was Pi-starved, and *AtPPC3* transcripts when plants were grown for 2 wk with 100 mM NaCl.

When cultivated with 5 μM CdCl_2 , seedlings that did not express *AtPPC1* produced less transcripts of *AtPPC3* compared to wild-type, and the seedlings that did not express *AtPPC3* produced less transcripts of *AtPPC1*. However, each of the mutant lines produced significantly higher *AtPPC2* transcript levels, perhaps as a compensatory response. A similar phenomenon occurred in response to drought; in mutants with reduced *AtPPC4* expression, the genes for the three other PEPC isozymes were up-regulated (Wang et al., 2012). More studies are required to determine whether this apparent compensatory gene expression is required for the survival of the mutant seedlings.

Gregory and co-workers (2009) reported a marked induction of *AtPPCK1* and *AtPPCK2* in Pi-deficient Arabidopsis; this up-regulation corresponded with increased *in vivo* phosphorylation of AtPPC1, both in cell culture and in seedlings. In Fe-deficient Arabidopsis, *AtPPCK1*, but not *AtPPCK2*, was induced in roots (Lan et al., 2012). In our study, transcripts for both *AtPPCK1* and *AtPPCK2* increased in response to Cd but relative amounts of *AtPPCK1* transcripts were lower in the roots of *atppc1* and *atppc3* relative to wild-type. In another study, *atppck1* mutants with reduced (*din1*) or no (*csi8*) light-induced PEPC phosphorylation had reduced rosette diameter and lower concentrations of succinate and fumarate, but no significant differences in glutamate, glutamine, or citrate and malate (Meimoun et al., 2009). This suggests that inhibition of *AtPPCK1* and subsequent reduced phosphorylation of PEPC may not affect the portion of the TCA cycle dedicated to amino acid synthesis.

PEPC-specific activity increased in response to Cd in roots and shoots of Arabidopsis and *atppc1* and *atppc3* had reduced root PEPC activity compared with wild-type plants. Similarly, Gouia et al. (2003) reported a 40 % increase in activity of PEPC in bean leaves, and a 50 % decrease in roots, in response to 5 μM CdCl_2 . In tomato (*Lycopersicon esculentum* L.) roots, but not leaves, PEPC activity also increased in response to 10 and 100 μM CdCl_2 (López-Millán et al., 2009). In roots and shoots of Pi-deficient Arabidopsis, an increase in PEPC phosphorylation corresponded with increased PEPC activity (Uhde-Stone et al., 2003; Gregory et al., 2009; Feria

et al., 2016). While *atppc1* and *atppc3* PPCK activity significantly increased in response to Cd, these activity levels were comparable to or less than what was observed in wild-type plants. Murmu and Plaxton (2006) provided evidence that PPCK could be controlled post-translationally by several metabolite effectors as well as by reversible dithiol-disulfide interconversion. It is also possible in wild-type plants that PPCK turnover rates increase in response to Cd-stress, which could explain the increase in root *AtPPCK1* and *AtPPCK2* transcripts and p107 phosphorylation but no measured increase in PPCK activity. Slower turnover rates in the shoots could account for the increased relative PEPC phosphorylation and PPCK activity compared to the roots.

4.3 Organic acid anions may mitigate Cd-induced damage

Initiation of the anaplerotic PEPC pathway followed by increased production of organic acid anions can help plants acclimate to abiotic stresses such as Pi deficiency or trace metal toxicity. The primary roles of the TCA cycle and mitochondrial respiration are the production of NADH and chemiosmotic generation of ATP (Fernie et al., 2004), and to generate the organic acid precursors needed for N-assimilation *via* GS/GOGAT and amino acid biosynthesis (Buchanan et al., 2000). The amino acids Glu, Cys and usually Gly are required to produce phytochelatins (Buchanan et al., 2000). All of these products may be used to repair damage or invest in defensive mechanisms.

Since organic acid anions are inherently linked to the chelation of Cd^{2+} and the repair of damaged cells, their abundance in roots and shoots would be expected to increase when a plant is exposed to Cd. In our study, concentrations of measured organic acid anions increased in roots in response to Cd. Several other studies have reported increased organic acid anion concentrations after exposure to Cd (Adeniji et al., 2010; Hawrylak-Nowak et al., 2015; Javed et al., 2017). However, even if the concentrations of organic acid anions remained at control values, the results cannot be interpreted as showing no up-regulation of anaplerotic PEP carboxylation mediated by PEPC. If the rate of replenishment matched the rate of consumption, then no net change in concentration would be detected.

Lebouteiller et al. (2007) reported increased malate in response to PTPC overexpression in *Arabidopsis*. In our study, upregulated PTPC expression and increased PEPC activity were coincident with increased concentrations of succinate, citrate and oxalate in Cd-treated roots. Others have also reported that increased organic acid anion content in roots is associated with increased Cd uptake. For example, exogenous malate and acetate resulted in about 33% more Cd in roots of sunflower (*Helianthus annuus* L.), along with decreased translocation of Cd to shoots and reduced symptoms of Cd toxicity (Hawrylak-Nowak et al., 2015). In addition, citrate- Zn^{2+} (which is similar in size and charge to Cd^{2+}) complexes were found in the xylem sap of the Zn-hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl.) (Salt et al., 1999). Organic acid anions can chelate and sequester metal ions but they can also act as antioxidants, thereby reducing the symptoms of toxicity. Hawrylak-Nowak et al. (2015) found that malate was better than citrate in terms of reducing the concentration of hydrogen peroxide in Cd-treated sunflower roots.

5. Conclusions

Our results are consistent with the hypothesis that exposure to Cd results in increased synthesis, activity, and phosphorylation of PTPC. PEPC collected from the roots and the shoots of *Arabidopsis* had two distinct responses to Cd. Shoots accumulated higher levels of phosphorylated PTPC but had lower PEPC activity compared to the roots. In contrast, the roots accumulated lower concentrations of dephosphorylated PTPC but had higher PEPC activity. Lines disrupting *AtPPC3* and to a lesser extent, *AtPPC1*, were more sensitive than wild-type plants to Cd, as evidenced by reduced rosette diameter and biomass. Mutants accumulated less phosphorylated PEPC and lower PEPC activity in roots. This indicates both *AtPPC1* and *AtPPC3* play an important role in mediating Cd stress.

Furthermore, we hypothesize that lower concentrations of phosphorylated PTPC could be a result of the rapid turnover of *AtPPC1* or *AtPPC3* in the Cd-injured roots. *Arabidopsis* grown with 5 μM CdCl_2 accumulated higher concentrations of succinate, citrate and oxalate acid anions

in the roots compared to plants grown in the absence of CdCl_2 . Higher concentrations of organic acid anion chelators could explain the higher concentrations of Cd in the root. A reduction in organic acid anions in roots of *atppc1* and *atppc3* grown with CdCl_2 could explain the decreases in biomass and rosette diameter relative to wild-type plants.

While concentrations of organic acid anions increased in response to Cd treatment, fully understanding the links between PEPC activity, organic acid synthesis and Cd tolerance will require more work. We also observed that the disruption of either *AtPPC1* or *AtPPC3* caused a compensatory increase in expression of the *ATPPC2* genes. It will be important to establish the amount and activity of specific AtPPC isozymes in stressed plants, use of mutants lacking one or more of the PTPCs would further clarify the relative importance of the various isozymes.

This research is relevant to the applied efforts of biotechnologists to engineer transgenic crops exhibiting an improved accumulation of toxic trace metals in non-edible organs or conversely the engineering of hyperaccumulating plants for improved soil phytoremediation.

Author statements

Ian R. Willick: Investigation, Validation, Analysis and interpretation of data, Writing – original draft, Writing – review and editing, Visualization. **William C. Plaxton:** Conceptualization, Resources (antibodies), Analysis and interpretation of data, Writing – review and editing, Funding acquisition. **Susan J. Lolle:** Validation of mutants, Analysis and interpretation of data, Writing – review and editing. **Sheila M. Macfie:** Conceptualization, Resources (all other reagents and materials), Analysis and interpretation of data, Writing – review and editing, Supervision, Project Administration, Funding Acquisition. All authors approved the manuscript.

Declarations of interest: none.

Acknowledgements

This work was supported by the Natural Science and Engineering Council of Canada [grant numbers 203616 (SMM) and 106349 (WCP)] and University of Waterloo (SJL). We thank Amanda Moehring, James Staples, Denis Maxwell, and Norm Hüner for providing access to equipment for the RNA and protein work; Marianna Krol, Rainer Bode, Allyson Hill, and Rachelle Kanippayoor for their technical advice; Serena Thamilselvan and Christina Doherty for mutant screening, Charlie Wu for ICP-AES analysis, and Thomas Ulanowski for RP-HPLC analysis.

References

- Adeniji, B., Budimir-Hussey, M.T., Macfie, S.M., 2010. Production of organic acids and adsorption of Cd on roots of durum wheat (*Triticum turgidum* L. var. *durum*). *Acta Physiol. Plant.* 32, 1063–1072.
- Akhter, M.F., Omelon, C.R., Gordon, R.A., Moser, D., Macfie, S.M., 2014. Localization and chemical speciation of cadmium in the roots of barley and lettuce. *Environ. Exp. Bot.* 100, 10–19.
- Aluru, M.R., Bae, H., Wu, D., Rodermel, S.R., 2001. The *Arabidopsis* *immutans* mutation affects plastid differentiation and the morphogenesis of white and green sectors in variegated plants. *Plant Physiol.* 127, 67–77.
- Buchanan, B., Gruissem, W., Jones, R., 2000. *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Maryland.
- Divi, U.K., Rahman, T., Krishna, P., 2010. Brassinosteroid-mediated stress tolerance in *Arabidopsis* shows interactions with abscisic acid, ethylene and salicylic acid pathways. *BMC Plant Biol.* 10, 151.
- Feria, A.B., Bosch, N., Sánchez, A., Nieto-Ingelmo, A.I., de la Osa, C., Echevarría, C., García-Mauriño, S., Monreal, J.A., 2016. Phosphoenolpyruvate carboxylase (PEPC) and PEPC-kinase (PEPC-k) isoenzymes in *Arabidopsis thaliana*: role in control and abiotic stress conditions. *Planta* 244, 901–913.
- Fernie, A.R., Carrari, F., Sweetlove, L.J., 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Curr. Opin. Plant Biol.* 7, 254–261.
- Fontaine, V., Hartwell, J., Jenkins, G.I., Nimmo, H.G., 2002. *Arabidopsis thaliana* contains two phosphoenolpyruvate carboxylase kinase genes with different expression patterns. *Plant Cell Environ.* 25, 115–122.
- Fukuda, N., Hokura, A., Kitajima, N., Terada, Y., Saito, H., Abe, T., Nakai, I., 2008. Micro x-ray fluorescence imaging and micro x-ray absorption spectroscopy of cadmium hyper-accumulating plant, *Arabidopsis halleri* ssp. *gemmifera*, using high-energy synchrotron radiation. *J. Anal. Atom. Spectrom.* 23, 1068–1075.

- Gouia, H., Suzuki, A., Brulfert, J., Ghorbal, M.H., 2003. Effects of cadmium on the co-ordination of nitrogen and carbon metabolism in bean seedlings. *J. Plant Physiol.* 160, 367–376.
- Grant, C.A., Buckley, W.T., Bailey, L.D., Selles, F., 1998. Cadmium accumulation in crops. *Can. J. Plant Sci.* 78, 1–17.
- Gregory, A.L., Hurley, B.A., Tran, H.T., Valentine, A.J., She, Y-M., Knowles, V.L., Plaxton, W.C., 2009. In vivo regulatory phosphorylation of the phosphoenolpyruvate carboxylase AtPPC1 in phosphate-starved *Arabidopsis thaliana*. *Biochem. J.* 420, 57–65.
- Hawrylak-Novak, B., Dresler, S., Matraszek, R., 2015. Exogenous malic and acetic acids reduce cadmium phytotoxicity and enhance cadmium accumulation in roots of sunflower plants. *Plant Physiol. Biochem.* 94, 225–234.
- Hétu, M-F., Tremblay, L.J., Lefebvre, D.D., 2005. High root biomass production in anchored *Arabidopsis* plants grown in axenic sucrose supplemented liquid culture. *Biotechniques* 39, 345–349.
- Igawa, T., Masayuki, F., Tanaka, I., Fukao, Y., Yanagawa, Y., 2010. Characterization of bacterial-type phosphoenolpyruvate carboxylase expressed in male gametophyte of higher plants. *BMC Plant Biol.* 10, 200.
- Javed, M.T., Akram, M.S., Tanwir, K., Javed-Chaudhary, H., Ali, Q., Stoltz, E., Lindberg, S., 2017. Cadmium spiked soil modulates root organic acids exudation and ionic contents of two differentially Cd tolerant maize (*Zea mays* L.) cultivars. *Ecotoxicol. Environ. Saf.* 141, 216–225.
- Kochian, L.V., Piñeros, M.A., Liu, J., Magalhaes, J.V., 2015. Plant adaptation to acid soils: the molecular basis for crop aluminum resistance. *Ann. Rev. Plant Biol.* 66, 571–598.
- Kutrowska, A., Szelag, M., 2014. Low-molecular weight organic acids and peptides involved in the long distance transport of trace metals. *Acta Physiol. Plant.* 36, 1957–1968.
- Lan, P., Li, W., Wen, T-N., Schmidt, W., 2012. Quantitative phosphoproteome profiling of iron-deficient *Arabidopsis* roots. *Plant Physiol.* 159, 403–417.
- Lebouteiller, B., Gousset-Dupont, A., Pierre, J-N., Bleton, J., Tchapla, A., Maucourt, M., Moing, A., Rolin, D., Vidal, J., 2007. Physiological impacts of modulating phosphoenolpyruvate carboxylase levels in leaves and seeds of *Arabidopsis thaliana*. *Plant Sci.* 172, 265–272.
- López-Millán, A.F., Sagardov, R., Solanas, M., Abadia, A., Abadia, J., 2009. Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. *Environ. Exp. Bot.* 65, 376–385.
- Macfie, S.M., Bahrami, S., McGarvey, B.D., 2016. Differential accumulation of cadmium in near-isogenic lines of durum wheat: no role for phytochelatins. *Physiol. Mol. Biol. Plant.* 22, 461–472.
- Maksymiec, W., Wójcik, M., Krupa, Z., 2007. Variation in oxidative stress and photochemical activity in *Arabidopsis thaliana* leaves subjected to cadmium and excess copper in the presence or absence of jasmonate and ascorbate. *Chemosphere* 66, 421–427.
- Martínez-Peñalver, A., Graña, E., Reigosa, M.J., Sánchez-Moreiras, A.M., 2012. The early response of *Arabidopsis thaliana* to cadmium- and copper-induced stress. *Environ. Exp. Bot.* 78, 1–9.
- Masakapalli, S.K., Bryant, F.M., Kruger, N.J., Ratcliffe, R.G., 2014. The metabolic flux phenotype of heterotrophic *Arabidopsis* cells reveals a flexible balance between the cytosolic

- and plastidic contributions to carbohydrate oxidation in response to phosphate limitation. *Plant J.* 78, 964–977.
- Meimoun, P., Gousset-Dupont, A., Lebouteiller, B., Ambard-Bretteville, F., Besin, E., Lelarge, C., Mauve, C., Hodges, M., Vidal, J., 2009. The impact of PEPC phosphorylation on growth and development of *Arabidopsis thaliana*: molecular and physiological characterization of PEPC kinase mutants. *FEBS Lett.* 583, 1649–1652.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant* 15, 473–497.
- Murmu, J., Plaxton, W.C., 2007. Phosphoenolpyruvate carboxylase protein kinase from developing castor oil seeds: partial purification, characterization, and reversible control by photosynthate supply. *Planta* 226, 1299–1310.
- Nocito, F.F., Espen, L., Crema, B., Cocucci, M., Sacchi, G.A., 2008. Cadmium induces acidosis in maize root cells. *New Phytol.* 179, 700–711.
- O'Leary, B., Fedosejevs, E.T., Hill, A.T., Bettridge, J., Park, J., Rao, S.K., Leach, C.A., Plaxton, W.C., 2011a. Tissue-specific expression and post-translational modifications of plant- and bacterial-type phosphoenolpyruvate carboxylase isozymes of the castor oil plant, *Ricinus communis* L. *J. Exp. Bot.* 62, 5485–5495.
- O'Leary, B., Park, J., Plaxton, W.C., 2011b. The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological functions and post-translational control of non-photosynthetic PEPCs. *Biochem. J.* 436, 15–34.
- Pocock, T., Król, M., Hüner, N.P.A., 2004. The determination and quantification of photosynthetic pigments by reverse phase high-performance liquid chromatography, thin-layer chromatography, and spectrophotometry. *Met. Mol. Biol.* 274, 137–148.
- Porra, R.J., Thompson, W.A., Kriedemann, P.E., 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* 975, 384–394.
- Rao, X., Huang, X., Zhou, Z., Lin, X., 2013. An improvement of the $2^{-\Delta\Delta CT}$ method for quantitative real-time polymerase chain reaction data analysis. *Biostat. Bioinforma. Biomath.* 3, 71–85.
- Rausser, W.E., 1999. Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin and metallothioneins. *Cell Biochem. Biophys.* 31, 19–38.
- Rioval, J., Trzos, S., Gage, D.A., Plaxton, W.C., Turpin, D.H., 2001. Two unrelated phosphoenolpyruvate carboxylase polypeptides physically interact in the high molecular mass isoforms of this enzyme in the unicellular green alga *Selenastrum minutum*. *J. Biol. Chem.* 276, 12588–12597.
- Salt, D.E., Prince, R.C., Baker, A.J.M., Raskin, I., Pickering, I.J., 1999. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. *Environ. Sci. Technol.* 33, 713–717.
- Sánchez, R., Cejudo, F.J., 2003. Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from *Arabidopsis* and rice. *Plant Physiol.* 132, 949–957.
- Sánchez, R., Flores, A., Cejudo, F.J., 2006. *Arabidopsis* phosphoenolpyruvate carboxylase genes encode immunologically unrelated polypeptides and are differentially expressed in response to drought and salt stress. *Planta* 223, 901–909.

- Sanità di Toppi, L., Gabbrielli, R., 1999. Response to cadmium in higher plants. *Environ. Exp. Bot.* 41, 105–130.
- Tripodi, K.E., Turner, W.L., Gennidakis, S., Plaxton, W.C., 2005. In vivo regulatory phosphorylation of novel phosphoenolpyruvate carboxylase isoforms in endosperm of developing castor oil seeds. *Plant Physiol.* 139, 969–978.
- Tsang, V., Peralta, J., Simons, A., 1983. Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Method. Enzymol.* 92, 377–391.
- Uhde-Stone, C., Gilbert, G., Johnson, J.M.F., Litjens, R.E., Zinn, K., Temple, S.J., Vance, C.P., Allan, D.L., 2003. Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. *Plant Soil* 248, 99–116.
- Uhrig, R.G., She, Y.M., Leach, C.A., Plaxton, W.C., 2008. Regulatory monoubiquitination of phosphoenolpyruvate carboxylase in germinating castor oil seeds. *J. Biol. Chem.* 283, 29650–29657.
- US EPA (United States Environmental Protection Agency), 2011. Test methods for evaluating solid waste, physical/chemical methods SW-846. <https://www.epa.gov/hw-sw846/sw-846-compendium> (accessed 21 January 2019).
- Vidal, J., Chollet, R., 1997. Regulatory phosphorylation of C4 PEP carboxylase. *Trends Plant Sci.* 2, 230–237.
- Walters, R.G., Horton, P., 1994. Acclimation of *Arabidopsis thaliana* to the light environment: changes in composition of the photosynthetic apparatus. *Planta* 195, 248–256.
- Wang, F., Liu, R., Wu, G., Lang, C., Chen, J., Shi, C., 2012. Specific downregulation of bacterial-type PEPC gene by artificial microRNA improves salt tolerance in *Arabidopsis*. *Plant Mol. Biol. Rep.* 30, 1080–1087.
- Xu, H-W., Ji, X-M., He, Z-H., Shi, W-P., Zhu, G-H., Niu, J-K., Li, B.S., Peng, X.X., 2006. Oxalate accumulation and regulation is independent of glycolate oxidase in rice leaves. *J. Exp. Bot.* 57, 1899–1908.