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## Absence of phosphoenolpyruvate carboxylase AtPPC3 increases sensitivity of *Arabidopsis thaliana* to cadmium

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science  
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ABSENCE OF PHOSPHOENOLPYRUVATE CARBOXYLASE AtPPC3  
INCREASES SENSITIVITY OF *ARABIDOPSIS THALIANA* TO CADMIUM

(Thesis format: Monograph)

by

Ian Robert Willick

Graduate Program in Biology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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## Abstract

Phosphoenolpyruvate carboxylase (PEPC) and PEPC kinase (PPCK) catalyze a reaction which feeds into the tricarboxylic acid (TCA) cycle and increases production of metal-chelating organic acids. Little research has been conducted on PEPC isoenzymes in Cd-stressed plants. *Arabidopsis* (*Arabidopsis thaliana*) wild-type and *Atppc1* – *Atppc3*, each lacking one of three PEPC isoenzymes, grown in 0, 1, or 5  $\mu\text{M}$   $\text{CdCl}_2$  were smaller and had increased *AtPPC1* – *AtPPC3* and *AtPPCK1* – *AtPPCK2* transcript abundance, relative phosphorylation, and PEPC activity, more so in roots than shoots. Concentrations of oxaloacetic, citric and total organic acids increased with greater  $\text{CdCl}_2$  concentrations. The absence of *AtPPC3*, and to a lesser extent *AtPPC2*, resulted in greater oxaloacetic acid concentrations and smaller plants in comparison with wild-type. My results indicate that *AtPPC3* plays an integral role in *Arabidopsis*' ability to cope with Cd. This information can be used to better understand Cd tolerance and stress in other plants, including crops.

## Keywords

Cadmium, immunoblotting, isoenzyme, organic acids, phosphoenolpyruvate carboxylase (PEPC), PEPC specific kinase (PPCK), plant-metal interactions, RT-qPCR, RP-HPLC.

## Co-Authorship Statement

This thesis includes one manuscript in preparation for publication co-authored by myself, Dr. Sheila M. Macfie and Dr. William C. Plaxton. I will be the first author on the publication. I designed and conducted all experiments, analyzed the data and will be writing the manuscript. Dr. Macfie provided laboratory support, financial assistance and guidance in experimental design, data interpretation and manuscript preparation. Dr. Plaxton in concert with Dr. Macfie developed the initial basis for the project, as well as provided training at Queen's University.

## Acknowledgments

First and foremost I need to express my sincere thanks to my supervisor Dr. Sheila Macfie for her support and acting as both a friend and mentor. Her advice on everything from experimental design to her positive encouragement has helped create the researcher that I am today. Sheila, I will always be indebted to you for opening your lab door for me. I am also grateful to my advisors Drs. Norm Hüner and Denis Maxwell for their guidance and patience. I must thank my unofficial advisor, Dr. Bill Plaxton for his kindness and generosity.

I would like to thank all the previous and current members of the Macfie lab for their support. To my “big sisters”, Shathi and Melanie for all your advice, guidance and coffee break pep talks. You constantly pushed me to be better than I thought I was. Nahed, thank you for always lightening the mood with your radiant smile. Joanna, who knew a friendship could form because of cabbage looper’s poo? I will always cherish our adventures in Staging and afternoon chats.

Thank you to my friends in NCB and the Moehring lab for befriending and adopting me into your labs.

I would like to thank the following people for technical support, service, the use of equipment, and free advice: Tom Ulanowski, Ally Hill, Dr. Charlie Wu, Dr. Marianna Krol, Rainer Bode, Ian Craig, Dr. Amanda Moehring, Dr. Jim Staples, Dr. Brent Sinclair, Elizabeth Myscich, and Rachelle Kanippayoor.

Mom and Dad, all of my successes in life are because of you. Your love and support have made me who I am today. Audrey and Steve I started this journey with you, I wish you were here to see how everything turned out. I have and always will love you.

Financial support for this project came from an NSERC Discovery Grant and the UWO Graduate Thesis Research Fund.

# Table of Contents

Abstract .....	ii
Keywords .....	ii
Co-Authorship Statement.....	iii
Acknowledgments.....	iv
Table of Contents .....	v
List of Figures .....	viii
List of Appendices .....	ix
List of Abbreviations .....	x
1 Introduction .....	1
1.1 Metals in the environment .....	1
1.2 Cd in the environment.....	1
1.3 Cd toxicity in plants .....	2
1.3.1 Measuring stress in plants.....	3
1.4 Mechanisms of defense against Cd toxicity.....	4
1.4.1 Proteins and polypeptides .....	5
1.4.2 Organic acids .....	6
1.5 Role of PEPC in organic acid production .....	9
1.6 Overview of PEPC .....	11
1.6.1 Post-translational control of PEPC .....	13
1.7 Characterizing the PEPC-Cd relationship using the Arabidopsis model system..	18
1.8 Thesis objectives and hypothesis .....	20
2 Methods.....	23
2.1 Plant material and growth conditions .....	23
2.2 Measures of Cd stress .....	24

2.2.1	Biomass.....	24
2.2.2	Rosette diameter.....	25
2.2.3	Cd concentration .....	25
2.2.4	Chlorophyll <i>a</i> fluorescence.....	26
2.2.5	Chlorophyll content .....	26
2.3	Cd-induced effect on the PEPC pathway.....	27
2.3.1	RNA extraction and quantification.....	27
2.3.2	Quantitative RT-PCR of PEPC and PPCK genes.....	28
2.3.3	PEPC crude protein extracts .....	29
2.3.4	PEPC semi-quantification with immunoblotting.....	30
2.3.5	PPCK crude extractions .....	30
2.3.6	PPCK assay.....	31
2.3.7	PEPC activity assay .....	31
2.3.8	Extraction of organic acids from tissue .....	32
2.3.9	Organic acid analysis using RP-HPLC.....	32
2.4	Statistical analysis.....	33
3	Results.....	34
3.1	Effect of Cd and AtPPC knockout on seedling development.....	34
3.2	Response of PEPC pathway to Cd-induced stress .....	38
4	Discussion .....	49
4.1	General overview .....	49
4.2	Did the lack of a PEPC isoenzyme affect plant health or plant response to Cd? .	49
4.3	How important is the PEPC pathway to reducing Cd stress?.....	52
4.4	Conclusions and future work .....	59
	References.....	63

Appendices.....	76
Curriculum Vitae .....	82



## List of Figures

Figure 1-1 Alternative pathways for phosphoenolpyruvate to feed into the tricarboxylic acid (TCA) cycle.....	10
Figure 1-2 Simplified representation of the C <sub>4</sub> and CAM cycles. ....	12
Figure 1-3 Multiple sequence alignment of AtPPC1-AtPPC3 isoenzymes.....	16
Figure 3-1 Growth measurements of Arabidopsis at different CdCl <sub>2</sub> treatments. ....	35
Figure 3-2 Photosynthetic measurements of Arabidopsis exposed to increasing concentrations of Cd .....	36
Figure 3-3 Concentration of Cd in shoots (top) and roots (bottom) in Arabidopsis.....	37
Figure 3-4 Relative quantification of <i>AtPPC1</i> – <i>AtPPC3</i> transcript abundance in Arabidopsis .....	39
Figure 3-5 Relative quantification of <i>AtPPCK1</i> and <i>AtPPCK2</i> transcript abundance in Arabidopsis .....	40
Figure 3-6 Immunoblots of IgG p107, IgG pSer-11 and IgG APS in Cd treated Arabidopsis .....	42
Figure 3-7 Relative phosphorylation of class-1 PEPC and PPCK activity in Cd- treated Arabidopsis .....	43
Figure 3-8 PEPC specific activity of Arabidopsis plant lines exposed to CdCl <sub>2</sub> .....	46
Figure 3-9 Organic acid concentrations in Arabidopsis plant lines exposed to CdCl <sub>2</sub> .....	47
Figure 4-1 Fates of TCA cycle intermediates (organic acids) in Arabidopsis.....	56
Figure 4-2 Model depicting response of wild-type versus <i>Atppc3</i> to Cd stress in Arabidopsis .....	61

## List of Appendices

Appendix A Composition of MS nutrient medium (Murashige and Skoog, 1962).....	76
Appendix B Arabidopsis PCR primers.....	77
Appendix C Bradford Assay: total protein concentrations from Arabidopsis tissue used for equal protein loading.....	78
Appendix D Percentage recovery of organic acids from digested standards.....	79
Appendix E Example of RT-qPCR amplification curve from <i>AtPPC1</i> and <i>UBQ10</i> in Arabidopsis shoots.....	80
Appendix F APS IgG trial to determine approximate assay time.....	81

## List of Abbreviations

ANOVA	Analysis of Variance
AtPPC	<i>Arabidopsis thaliana</i> PEPC isoenzyme
AtPPC	<i>Arabidopsis thaliana</i> PEPC isoenzyme mRNA transcript
Atppc	<i>Arabidopsis thaliana</i> PEPC isoenzyme mutant line
Cd <sup>2+</sup>	Cadmium ion
Cd	Elemental cadmium
F <sub>v</sub> /F <sub>m</sub>	Photochemical efficiency of photosystem II
F <sub>m</sub>	Maximum fluorescence
F <sub>o</sub>	Minimum fluorescence
F <sub>v</sub>	Variable fluorescence
HPLC	High performance liquid chromatography
ICP-AES	Inductively coupled plasma-atomic emission spectroscopy
MDH	Malate dehydrogenase
ME	Malic enzyme
MS	Murashighe and Skoog
NIST	National Institute of Standards and Technology
PK <sub>c</sub>	Pyruvate kinase c
PP2A	Protein phosphatase 2A
PEPC	Phosphoenolpyruvate carboxylase
PPCK	Phosphoenolpyruvate carboxylase kinase
PS	Photosystem
RO	Reverse osmosis
Rpm	Revolutions per minute
TCA	Tricarboxylic acid cycle

# 1 Introduction

## 1.1 Metals in the environment

Both terrestrial and aquatic systems contain many metallic elements. Concentrations of essential metals, such as zinc (Zn) and magnesium (Mg), are required as enzyme co-factors for various plant processes including auxin and carbohydrate metabolism, protein and amino acid synthesis as well as in chlorophyll production (Hopkins and Hüner, 2009). In contrast, at high concentrations essential metals can become detrimental to plant health. For example, if Zn concentrations exceed their required amount then the plant can be damaged, leading to stunted growth and leaf chlorosis through disruption and disintegration of membranes and organelles as well as reduced function of sensitive enzymes (Sresty and Rao, 1999). High concentrations of essential metals can lead to plant cell death (Ingestad and Agren 1988). This phenomenon of metal toxicity extends to a group of metals that are not thought to be required by plants, but will readily accumulate in tissue. These metals include mercury (Hg), lead (Pb) (Godbold and Hüttermann, 1986), aluminum (Al) (Begum et al., 2009), chromium (Cr) (Prado et al., 2010), silver (Ag) (Oukarroum et al., 2013), and cadmium (Cd) (Page et al., 1981).

## 1.2 Cd in the environment

Of the non-essential metals, Cd is a significant pollutant in Canadian soils (Sheppard et al., 2007) and one of the four metals that are of world-wide concern due to their negative impact on environmental health (Sanità di Toppi and Gabbrielli, 1999). Concentrations of Cd have increased in recent years due to anthropogenic factors including combustion of fossil fuels, soil runoff, mining and smelting, improper disposal of industrial effluents as well as the use of phosphate-based fertilizers (Williams and David, 1976; McLaughlin and Singh, 1999; He et al., 2005). These fertilizers can contain Cd impurities ranging from trace amounts up to 340 mg/kg that were not removed during the fertilizer's refining from phosphate rocks (Alloway and Steinnes, 1999). Repeated use of contaminated fertilizers has led to Cd accumulation in agricultural soils in Canada (Sheppard et al., 2007). While Canada does limit the acceptable concentration of Cd in agricultural soils to

1.4 mg/kg (Canadian Council of Ministers of the Environment, 2007), this standard does not take into account the proportion available to, or taken up by, crop plants. Factors such as soil pH (Muhammad et al., 2012), microbial activity (Bollag and Czaban, 1989), organic matter content (Murray et al., 2011) and concentration of plant exudates such as organic acids (Costa and Michaut, 1997) can influence Cd solubility and, hence, Cd<sup>2+</sup> bioavailability.

Cd accumulation in agricultural systems and consumption of contaminated crops poses a potential human health risk. According to the United States Environmental Protection Agency (2000) Cd can accumulate in the kidneys, lungs and liver, and cause developmental abnormalities; Cd is categorized as a human carcinogen by the International Agency for Research on Carcinogens (2011). Furthermore Cd<sup>2+</sup> can replace calcium (Ca<sup>2+</sup>) in bones resulting in increased potential for bone fractures and early onset osteoporosis (Christoffersen et al., 1988).

### 1.3 Cd toxicity in plants

The toxic effect of Cd in plants is primarily due to its similarity to other elements present in group 12 of the periodic table of elements. In natural environments, the most stable valence state is Cd (II) (Baes and Mesmer, 1976). When ionized, Cd<sup>2+</sup> has an atomic radius similar to Zn<sup>2+</sup> and Ca<sup>2+</sup>. This allows Cd<sup>2+</sup> to compete with Ca<sup>2+</sup> for Ca<sup>2+</sup>- channels near guard cells, resulting in stomatal closure and decreased cellular gas exchange (Perfus-Barbeoch et al., 2002). Competition by Cd<sup>2+</sup> for membrane transporters can also result in a decrease in the relative uptake of Zn<sup>2+</sup>, leading to Zn deficiency (Pinto et al., 2004). In soil solution, Cd<sup>2+</sup> can co-precipitate with phosphate (PO<sub>4</sub><sup>2-</sup>), leading to phosphorus (P) deficiency (Lambert et al., 2007).

In addition to these indirect effects, Cd can directly inhibit plant growth and development. Cd first comes in contact with plants at the roots. High concentrations of unbound Cd<sup>2+</sup> in the apoplast can damage cells in the root tips resulting in increased membrane permeability (Dauthieu et al., 2009), as well as stunted (Dong et al., 2005) and browning roots (Liu et al., 1995). Cd enters the symplast via ZIP (Zn/Fe regulated

transporter-like proteins) transporters (Cohen et al., 1998; Pedas et al., 2008) or  $\text{Ca}^{2+}$  channels (Clemens et al., 1998) in the root membrane. Plants are able to limit root to shoot Cd translocation often leading to greater Cd concentrations in the roots (Lux et al., 2011), such as through absorption of  $\text{Cd}^{2+}$  into cell walls (Cosio et al., 2005).

The first visible symptoms of Cd toxicity to appear in shoot tissue include leaf rolling and chlorosis (Cosio et al., 2005). Internally,  $\text{Cd}^{2+}$  can disrupt photosystem (PS) I (Wiegel, 1985, Siedlecka and Baszynski 1993) and PS II (Krupa 1988). When bread wheat (*Triticum aestivum*) was treated with solution containing  $\text{Cd}^{2+}$ , both PS II and to a lesser extent PS I was negatively impacted (Atal et al., 1991). In PS II,  $\text{Cd}^{2+}$  interferes with electron flow by displacing  $\text{Mn}^{2+}$  co-factors, resulting in the disassociation of oxygen-evolving complex proteins (Baszynki et al., 1980; Prasad et al., 1991). Cd can also affect photosynthesis by causing a lowered total chlorophyll content, as has been measured in a number of species including rapeseed (*Brassica napus*; Larsson et al., 1998), cucumber (*Cucumis satvus*; Zhang et al., 2003), barley (*Hordeum vulgare*; Wu et al., 2003) and corn (*Zea mays*; Jain et al., 2007).

Siedlecka and Baszynski (1993) discovered that 10, 20 or 30  $\mu\text{M}$  Cd treatments resulted in decreased Fe and ferredoxin content in corn seedlings' chloroplasts. Cd reacts with Fe in ferredoxin altering the cell's ability to regenerate ribulose-1,5-bisphosphate. Inhibition of ferredoxin's ability to accept electrons decreases the cell's ability to create reducing power in the form of NADPH (Siedlecka and Baszynski, 1993). This leads to a down-regulation of 3-phosphoglycerate reduction to glyceraldehyde-3-phosphate.

Glyceraldehyde-3-phosphate is essential in the regeneration of ribulose-1,5-bisphosphate as well as for its roles in structural support and energy. In addition, glyceraldehyde-3-phosphate feeds into the stroma hexose phosphate pool resulting in the formation of starch or, when exported into the cytosol, it can feed into the sucrose biosynthetic pathway.

### 1.3.1 Measuring stress in plants

Measuring photochemical efficiency of PS II is a non-invasive technique used to determine a plant's ability to tolerate environmental stresses and determine the extent of

damage to PS II. Light energy absorbed by chlorophyll molecules in the light harvesting complex are used in PS II photochemistry, dissipated as heat (also known as non-photochemical quenching), or re-emitted as fluorescence (Maxwell and Johnson, 2000). An overabundance of light can damage the light harvesting complex. To avoid this damage the plant emits the excess energy in the form of heat or as fluorescence.

When PS II absorbs light and the electron carrier  $Q_A$  has accepted an electron,  $Q_A$  is unable to accept another electron until the first has passed onto  $Q_B$  and down the electron transport chain. When  $Q_A$  is reduced then the PS II reaction center is considered to be closed. The proportion of closed reaction centers leads to a decrease in the efficiency of photochemistry. When dark adapted for a period of time, electrons are drained from the photosynthetic electron transport chain by PS I causing all PS II reaction centers to be classified as open (Maxwell and Johnson, 2000). The minimum fluorescence yield is measured when all PS II reaction centers are open; this is called the background or initial fluorescence ( $F_o$ ). If the plant was exposed to a pulse of light capable of inducing photosynthesis, the subsequent fluorescence signal would be the maximum fluorescence ( $F_m$ ). The difference between the maximum and initial fluorescence is defined as variable fluorescence ( $F_v = F_m - F_o$ ). Therefore, measurements of the photochemical efficiency of PS II allow scientists to assess changes in the maximum efficiency of photochemistry and non-photochemical quenching in PS II. Photoinhibition as a result of low light or Cd-induced damage to the photosynthetic apparatus could be assessed by measuring the maximum photochemical efficiency of PS II ( $F_v/F_m$ ). Cd-induced stress could result in damage to the PS II reaction centers, disrupt electron transport or inhibit photochemistry, any of which would result in a decrease in the  $F_v/F_m$  ratio.

## 1.4 Mechanisms of defense against Cd toxicity

Plants have evolved different immobilization and sequestration strategies in order to limit cell damage caused by Cd. To minimize uptake, plants can exude chelators that can bind  $Cd^{2+}$  in the rhizosphere reducing its bioavailability. For example, when exposed to  $Cd^{2+}$  there were increased concentrations of amino acids and organic acids in root exudates of lettuce (*Lactuca sativa*), white lupin (*Lupinus albus*; Costa and Michaut, 1997) and

mangrove (*Kandelia obovata*; Xie et al., 2013). Plants can also exude molecules that alter the soil's pH (Taylor, 1988) or redox state (Bais et al., 2006) to alter the solubility of metal cations. Changes to the rhizosphere, including the exudation of sugars, might attract microbes that aid in the sequestration of Cd. Columbus and Macfie (unpublished data) discovered that unique rhizobacterial communities develop depending upon the Cd<sup>2+</sup> concentrations in the environment and the plant's ability to accumulate Cd. Mycorrhizal fungi have also been shown to form mutualistic relationships with plant roots in exchange for a carbon source. The fungi, in turn, form a hyphal network on the root's surface and extend out into the soil, where the fungi can bind with, and reduce the concentration of, metal ions around the plant's roots (Göhre and Paszkowski, 2006).

Internally, plants sequester metals in order to minimize damage. One such mechanism is through the chelation of metals by ligands and subsequent compartmentalization of metal-ligand complexes. The International Union of Pure and Applied Chemistry (1997) defines chelation as the formation of two or more coordinate bonds between one or more free metal ions and two or more ligands. Plants produce a number of metal-chelating molecules, which can be exuded into the rhizosphere or stored intracellularly. Three major types of organic compounds have recently been studied for their metal binding properties: metallothioneins, phytochelatins and its precursor glutathione, and organic acids.

#### 1.4.1 Proteins and polypeptides

Metallothioneins are small cysteine-rich proteins that are products of mRNA translation induced by toxic metal stress (Cobbett and Goldsbrough, 2002). Metals form chelation complexes with metallothionein by binding to the thiol groups on their cysteine peptides. While there has been extensive research conducted on metallothionein in mammals (Klaassen et al., 2009; Sabolić et al., 2010) and fish (Yudkovski et al., 2008; Ghedira et al., 2010), research on metallothionein in plants has been minimal. Tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) seedlings containing a metallothionein gene insert displayed a significant increase in Cd uptake as well as tolerance to increased intracellular Cd concentrations (Eapen and D'Souza, 2005). Clendennen and May (1997)



discovered an increase in metallothionein transcript abundance in ripening banana (*Musa acuminata*) fruit; however, its primary role has not yet been determined.

Phytochelatins are thiol-rich peptides that bind with free  $\text{Cd}^{2+}$  within plant tissues. There are multiple metal ions that can activate phytochelatin production; however, the strongest of those observed was  $\text{Cd}^{2+}$  (Grill et al., 1989). Phytochelatins are composed of three amino acids; glutamine, cysteine, and glycine (Rauser 1990). Phytochelatin production from glutathione ( $\gamma$ -glutamine-cysteine-glycine) is a self-regulated pathway. Metals act as a catalyst for phytochelatin synthase (EC 2.3.2.15) activity (Zhu et al., 1999) resulting in the formation of the general phytochelatin structure,  $(\gamma\text{-glutamine-cysteine})_n\text{-glycine}$ , where  $n$  ranges from 2 – 11 (Rauser 1990). Once the phytochelatin-metal complex forms, the metal is no longer available to act as a catalyst resulting in a decrease in phytochelatin synthase activity. Chelation of  $\text{Cd}^{2+}$  in roots by phytochelatins (Akhter et al., 2012) is typically followed by transport into the vacuole. Entry into the vacuole is facilitated by ABC transporters such as the so-called ‘heavy metal’ transporters (HMT1 and HMT2) in *Schizosaccharomyces pombe* (Prévéral et al., 2009). Metallothioneins and phytochelatins are functionally similar in that metals bind to free thiol groups.

#### 1.4.2 Organic acids

In contrast to phytochelatins and metallothioneins, organic acids chelate metals both externally and internally to the plant. The organic acids that are thought to play a role in the response to metal toxicity are low molecular weight tricarboxylic acid cycle (TCA) intermediates produced in the mitochondria and cytosol. One  $\text{Cd}^{2+}$  can form a chelation complex with two organic acid molecules (Lebouteiller et al., 2007).

Due to the nature of the TCA cycle, its organic acid intermediates are typically found in high concentrations in vacuoles and to a lesser extent in the mitochondria (López-Bucio et al., 2000). The relative amounts of each organic acid vary with plant species, age, and type of tissue (López-Bucio et al., 2000). Plants also have a relatively high abundance of organic acids in comparison with other organisms due to their function in metabolism (Plaxton and Podestá, 2006), role as photosynthetic intermediates (Chollet et al., 1996),

ability to balance excess cations in the cytosol, and their role as metabolically active solutes for osmotic adjustment (Oikawa et al., 2011). Not only are organic acids intermediates in the respiratory pathway, but organic acids can act as a substrate in both carbon and nitrogen assimilation (Plaxton and Podestá, 2006).

In the rhizosphere, organic acids can play a dual role. On one hand, chelation can increase metal solubility in the soil solution, which will facilitate the uptake of essential metals and prevent precipitation of absorbed nutrients (Cosio et al., 2005). In both tobacco and mapacho (*Nicotiana rustica*), known Cd-accumulators, an increase in organic acid exudates corresponded with increased Cd uptake (Mench and Martin, 1991). This suggests that the presence of organic acid can increase the solubility of  $\text{Cd}^{2+}$  in the rhizosphere. However, plants can also use organic acids to chelate metals, including  $\text{Cd}^{2+}$ , to reduce the toxicity associated with free metal ions (Perfus-Barbeoch et al., 2002). Exudation of chelators, including organic acids, usually results in Cd-organic acid complexes that are chemically inert and effectively removes  $\text{Cd}^{2+}$  from circulation (Pinto et al., 2004). Internally, plants sequester chelated Cd-organic acid complexes in vacuoles (Cosio et al., 2005).

Chelators such as organic acids have been well studied in relation to Al accumulation and most of these experiments have centered on research studying high and low Al-accumulating crop species. It has been determined that the exudation of organic acids from roots detoxifies soil  $\text{Al}^{3+}$  but the capacity of different organic acids to form stable complexes differs among plant species (Ryan et al., 2001). Of the TCA cycle organic acids, citric, malic and oxaloacetic are readily exuded and form stable complexes with  $\text{Al}^{3+}$  (Delhaize et al., 1991; Zheng et al., 1998); however, the exudation of organic acids in response to  $\text{Al}^{3+}$  appears to be species-specific. For example, citric acid is the predominant organic acid exuded from common bean (*Phaseolus vulgaris*; Miyasaka et al., 1991) and corn (Pellet et al., 1995), malic is predominant in bread wheat (Delhaize et al., 1991), and oxaloacetic is commonly exuded from rice (*Oryza sativa*; Begum et al., 2009) and buckwheat (*Fagopyrum esculentum*; Ma et al., 1997). Begum et al. (2009) determined that  $\text{Al}^{3+}$  soil amendments resulted in a significant increase in oxaloacetic, malic and citric acid concentrations in leaf tissues as well as increases in malic and citric

acid concentrations in root tissues. In both the wild type and transgenic rice lines that over-express C<sub>4</sub> phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) the total organic acid content increased in both shoot and root tissues (Begum et al., 2009).

The role of organic acids in chelation of other metal ions has also been studied. Increases in concentrations of citric acid in the vacuole of the hyper-accumulator *Thlaspi goesingense* corresponded with an increase in nickel (Ni) accumulation in shoot and root tissue (Krämer et al., 2000). In a species more sensitive to Ni toxicity, ryegrass (*Lolium perenne*), there was a 2 to 6 times greater concentration of organic acids in shoot xylem exudates in Ni-treated plants as compared to untreated plants (Yang et al., 1997). This suggests that organic acids play a role in Ni translocation. When Zn- and Cd-tolerant tussock grass (*Deschampsia caespitosa*) lines were treated with varying concentrations of Ni, Zn, copper (Cu), or mercury (Hg), only Zn treatment resulted in an increase in exudation of citric and malic acid (Thurman and Rankin, 1982). While pea (*Pisum sativum*) plants treated with Zn<sup>2+</sup> experienced stunted growth, the formation of Zn-succinate complexes decreased translocation into shoots (Doncheva et al., 2001). Mucha et al. (2010) determined that increasing Cu concentrations in media resulted in an increase in oxaloacetic acid in root exudates from sea rush (*Juncus maritimus*) roots. Furthermore, the ability of these plants to exude organic acids and take up Cu<sup>2+</sup> varied with the season (Mucha et al., 2010).

Exposure to Cd also results in increased root exudation of organic acids as well as increased internal concentrations. Cieslinski et al. (1998) determined that high Cd-accumulating cultivars of bread wheat had increased organic acid root exudation when compared to low accumulators (Cieslinski et al., 1998). Although, concentrations of organic acids in plant tissues do not necessarily follow this pattern. For example, Adeniji et al. (2010) found high concentrations of oxaloacetic acid in both the high and low accumulating lines of durum wheat (*Triticum durum*) and one of the durum wheat low Cd-accumulating lines had higher concentrations of organic acid than the high accumulating lines. In contrast, when treated with Cd<sup>2+</sup>, there was an increase in organic acids in the roots of corn (Guo et al., 2007) and mangrove (Weng et al., 2012), as well as

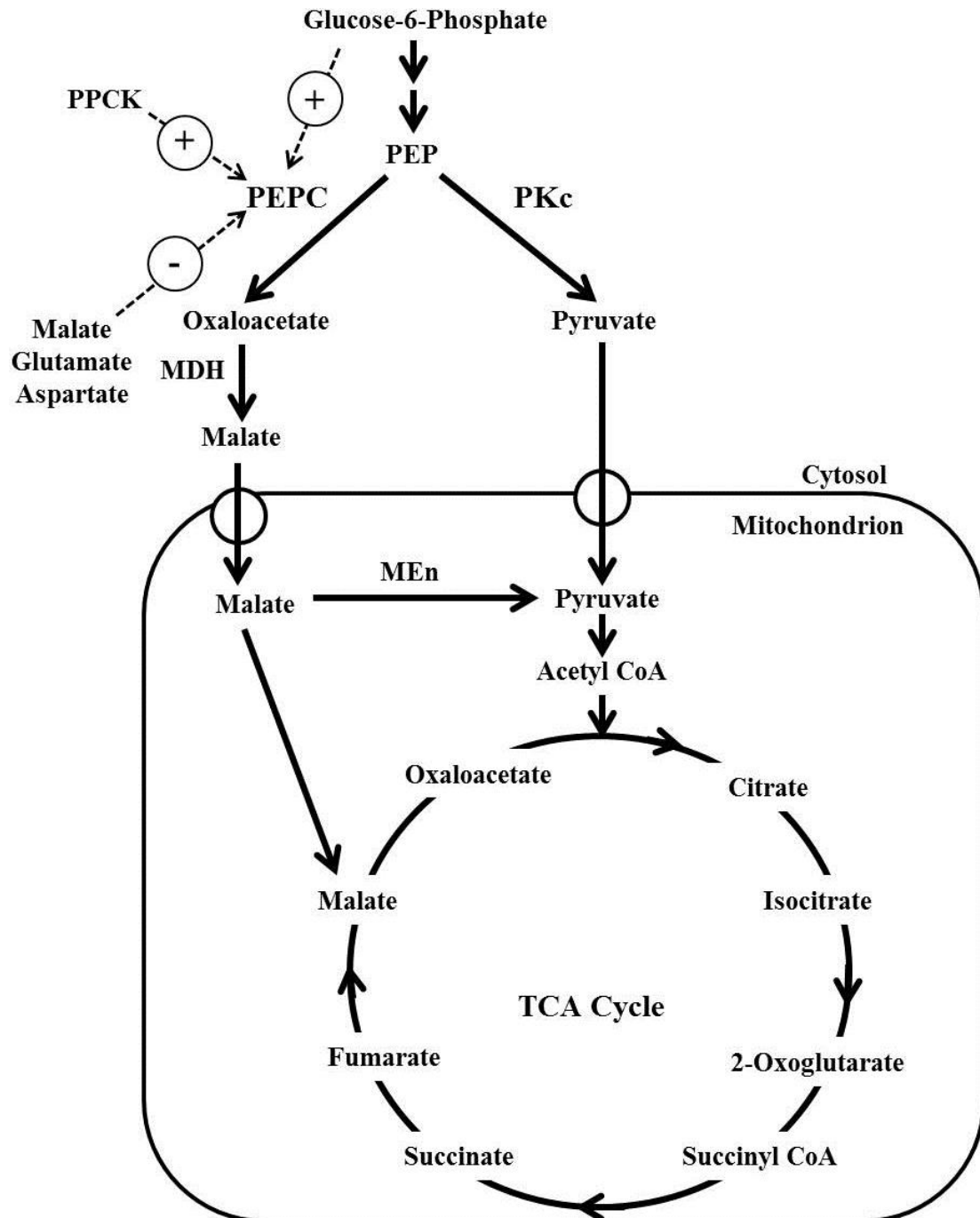
an increase of malic and citric acid in both black nightshade (*Solanum nigrum*) and tomato (*Solanum lycopersicum*) exudates (Bao et al., 2011).

The addition of exogenous organic acids has been used to chelate metals in the environment, reducing their solubility and uptake. For example, amendment of soil with citric acid resulted in a decrease in lead (Pb) toxicity and a decrease in the relative uptake of Cd into the roots and shoots in radish (*Raphanus sativus*) but did not relieve the physical symptoms of Cd toxicity (Chen et al., 2003). It is apparent that the relationship between specific organic acids and metal solubility varies based upon the toxic metal in question, the plant species and, possibly, soil chemistry.

## 1.5 Role of PEPC in organic acid production

Chelating organic acids are produced as intermediates in the TCA cycle (Fig 1.1). Under conditions where plants are supplied with sufficient nutrients, pyruvate kinase c (PK<sub>c</sub>; EC 2.7.1.40) catalyzes the dephosphorylation of phosphoenopyruvate resulting in the transfer of one P<sub>i</sub> to ADP and the formation of pyruvate and ATP (Plaxton and Podestá, 2006). Decarboxylation of pyruvate will produce Acetyl CoA, which feeds into the TCA cycle.

It should be noted that the TCA cycle does not always function as a continuous circle. Flux through one portion of the pathway may be low, while another portion might be high. In nutrient deficient or toxic metal environments, an anapleurotic reaction catalyzed by PEPC is up-regulated (Fig 1.1). In phosphorus (P<sub>i</sub>) deficient chickpea (*Cicer arietinum*), tomato, bread wheat (Neumann and Römheld, 2000), Arabidopsis (Gregory et al., 2009) as well as Fe deficient sugar beet (*Beta vulgaris*; López-Millán et al., 2001) increased PEPC activity resulted in a subsequent increase in concentrations of organic acids. Cd toxicity resulted in increased PEPC activity and malic acid production in corn roots (Nocito et al., 2008) but no other organic acids were measured in that study. The purpose of the PEPC pathway might be to restock the TCA cycle organic acids during periods of environmental stress.



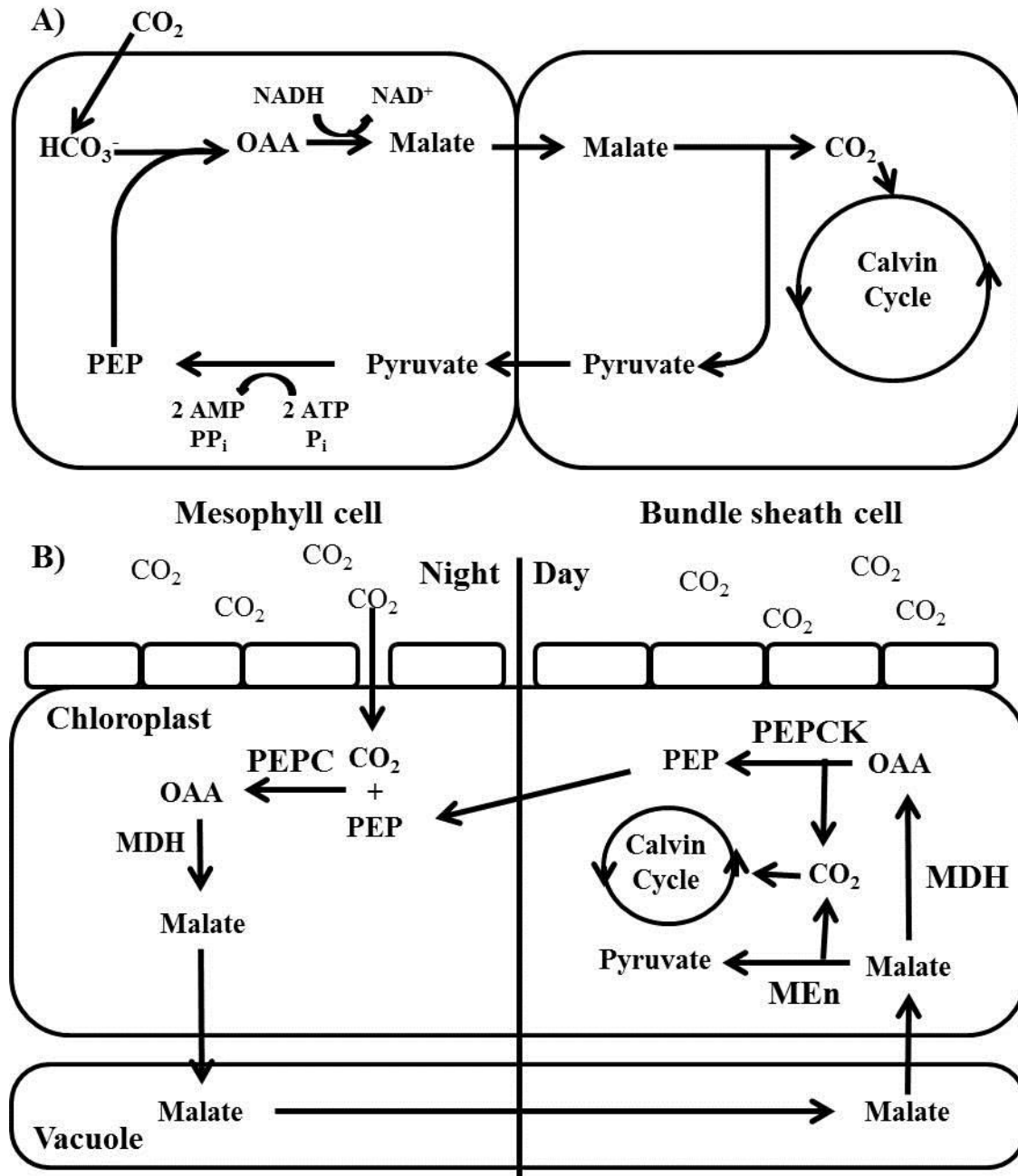
**Figure 1-1 Alternative pathways for phosphoenolpyruvate to feed into the tricarboxylic acid (TCA) cycle.**

Phosphoenolpyruvate (PEP), the product of glycolysis, can be converted to pyruvate by pyruvate kinase c (PKc) or converted to oxaloacetate by phosphoenolpyruvate carboxylase (PEPC) and then to malate by malate dehydrogenase (MDH). Allosteric effectors as well as phosphorylation by PEPC kinase (PPCK) can alter the activity level of PEPC. Other abbreviations include: malic enzymes (MEn) and co-enzyme A (coA). Modified from Plaxton and Podestá (2006).

## 1.6 Overview of PEPC

PEPC is an important enzyme in plant primary metabolism; it is present in all plants, green algae, cyanobacteria, most Archaea and non-photosynthetic bacteria, but as of yet has not been discovered in animals or fungi (Izui et al., 2004). It catalyzes the  $\beta$ -carboxylation of phosphoenolpyruvate in the presence of  $\text{HCO}_3^-$  and a  $\text{Mg}^{2+}$  co-factor, yielding oxaloacetate and inorganic phosphate ( $\text{P}_i$ ). Oxaloacetate is subsequently converted to malate by malate dehydrogenase (MDH; EC 1.1.1.37). In  $\text{C}_3$  plants, malate can undergo one of two fates: either react with a malic enzyme resulting in the production of  $\text{CO}_2$  and pyruvate or it can immediately feed into and help restock TCA cycle intermediates (Fig.1.1).

In plants utilizing  $\text{C}_4$  or Crassulacean acid metabolism (CAM) photosynthesis, PEPC is a rate-limiting enzyme involved in the fixation of atmospheric  $\text{CO}_2$  to phosphoenolpyruvate resulting in the formation of oxaloacetate and eventually malate.  $\text{C}_4$  plants carry out light-independent interactions in the bundle sheath cells (Fig. 1.2A). Malate is transported from the mesophyll to a bundle sheath cell where it is decarboxylated. The  $\text{CO}_2$  is fed into the Calvin cycle while pyruvate is recycled into phosphoenolpyruvate and continues in the cycle. CAM photosynthesis is an adaptation for increased water efficiency and is found in plants from arid regions. CAM plants operate on a day: night cycle (Fig.1.2B). At night, the plant's stomata open allowing for  $\text{CO}_2$  uptake and other gas exchange. Carbon is fixed in a similar fashion as with  $\text{C}_4$  plants but malate is stored within vacuoles. During the daytime, stomata close and CAM plants rely on their sequestered malate pools as a carbon source. Malate is oxidized by MDH or a malic enzyme (EC 1.1.1.38, EC 1.1.1.39 or EC 1.1.1.40) resulting in the formation of oxaloacetate or pyruvate, respectively. Free carbon arising from the production of pyruvate is fed into the Calvin cycle.



**Figure 1-2 Simplified representation of the  $C_4$  and CAM cycles.**

In  $C_4$  (A) and CAM (B) cycles phosphoenolpyruvate carboxylase (PEPC) catalyzes the  $\beta$ -carboxylation of phosphoenolpyruvate (PEP) from a three to four carbon molecule. Oxaloacetate (OAA) and malate are used to transfer carbon to the Calvin Cycle. In CAM plants stomata open at night allowing for gas exchange and the uptake of  $CO_2$ . Malate is stored until the daytime when stomata are closed. Malate is decarboxylated, and the excess carbon is fed into the Calvin cycle. Abbreviations include: malate dehydrogenase (MDH), pyruvate kinase c (PKc), phosphoenolpyruvate carboxykinase (PEPCK) and malic enzymes (MEn). Drawn using information from Plaxton and Podestá (2006).

### 1.6.1 Post-translational control of PEPC

Like most enzymes, PEPC activity is subject to allosteric regulation. Precursors of phosphoenolpyruvate, glucose-6-phosphate and triose phosphates, act as positive metabolite effectors while malate, aspartate, and glutamate, inhibit PEPC activity. Aspartate and glutamate are additional feedback effectors of PEPC and PKc in green algae and plant tissues involved in nitrogen assimilation (Plaxton and Podestá, 2006). This provides a regulatory link between nitrogen metabolism and the control of respiratory carbon metabolism. During periods of enhanced N-assimilation, glutamate concentrations decrease and can no longer act as an allosteric inhibitor of both PEPC and PKc. In contrast with its role in PEPC regulation, aspartate activates PKc and can relieve the enzyme of its glutamate inhibition (Turner and Plaxton, 2000; Turner et al., 2005). Allosteric control by aspartate and glutamate have been documented in green algae (Huppe and Turpin, 1994), spinach (*Spinacia oleracea*; Baysdorfer and Bassham, 1984), ripening banana fruit (Turner et al., 2005), as well as castor oil plant (*Ricinus communis*; Blonde and Plaxton, 2003) leaves and developing endosperm. This relationship between PEPC and PKc activation may occur when the cell contains an adequate amount of N and the rate of protein synthesis becomes dependent upon ATP and not on greater amino acid concentrations. In a non-stressed environment, plant respiration may assume a greater role in satisfying ATP demand, rather than the generation of organic acid precursors.

Since its discovery in the 1980s, regulation of PEPC by phosphorylation has been documented in Arabidopsis, castor oil plants and green algae to name a few. The site of phosphorylation was first discovered in CAM and C<sub>4</sub> photosynthetic PEPC as a single, highly conserved serine (Ser) residue near the N-terminus (Nimmo et al., 1986; Jiao et al., 1991). This post-translational modification of PEPC results in enzyme activation by decreasing PEPC's sensitivity to allosteric inhibitors such as malate, glutamate or aspartate, while increasing its affinity to activators such as PEP precursors. This reversible phosphorylation event is carried out by a Ca-independent PEPC specific kinase (PPCK; EC: 4.1.1.49) while dephosphorylation is controlled by a protein phosphatase (PP2A; EC 3.1.3.16; Tripodi et al., 2005)



PEPC phosphorylation status appears to be generally controlled by changes in the rates of PPCK synthesis versus degradation (Marsh et al., 2003; Izui et al., 2004). This is considered to be a novel means of control, considering that most protein kinases are post-translationally controlled by secondary messengers such as  $\text{Ca}^{2+}$  ions or by phosphorylation by other protein kinases (Plaxton and Podestá, 2006). PPCK activity is up-regulated by the presence of light in  $\text{C}_4$  plants; during the night phase of the circadian rhythm in CAM leaves; and light, N supply and Pi deprivation in  $\text{C}_3$  plants (Chollet et al., 1996). In castor oil plants, PPCK activity and phosphorylated subunits were undetected when plants were placed in the dark but the phosphorylated subunits were detectable following re-illumination (Uhrig et al., 2008). These results indicate a direct relationship between the up-regulation of PPCK and PEPC phosphorylation following the continuation of photosynthetic delivery of  $\text{CO}_2$  from illuminated leaves to non-photosynthetic tissues. Some post-translational controls have been proposed for PPCK including malate inhibition (Law and Plaxton, 1997; Murmu and Plaxton, 2007), thiol-disulfide interconversion (Saze et al., 2001), or a proteinaceous inhibitor (Nimmo et al., 2001). However, these mechanisms for control still require more research. Additional research is also required to assess the mechanisms of PP2A regulation under nutrient deficient or metal toxic conditions.

According to the seminal research done by Hunter and Merkert (1957), enzymes that differ in amino acid sequence but catalyze the same chemical reaction are classified as isoenzymes. In order to be classified as an isoenzyme, the enzyme variants must be products of different genes or the product of different alleles of the same gene. Isoenzymes evolved from mutations such as gene duplication, polyploidisation, or nucleic acid hybridization. If the activity or transcript abundance of the protein copy is modified by mutations, then the two isoenzymes will, over time, specialize in function (Hartmann et al., 2003). For example, one isoenzyme may become more prevalent in shoot tissue while the other in root tissue. However if the isoenzymes remain identical in both activity and transcript abundance, then one of the two copies will cease to function due to accumulated mutations (Hartmann et al., 2003). This will result in the formation of a pseudogene. At least four PEPC isoenzymes have been identified in plants.

All PEPC molecules discovered to date are either plant type PEPC or bacterial type PEPC. Bacterial type PEPCs share slightly higher similarity with PEPCs from proteobacteria than with the plant type PEPC (Sanchez and Cejudo, 2003). All currently discovered bacterial type PEPC and plant type PEPC subunits have distinguishable characteristics that separate one from the other. Plant type PEPC contains 10 highly conserved exons, while bacterial type PEPC contains 20 (Izui et al. 2004). The bacterial type PEPC polypeptides range in size from 116-118 kDa while the plant type PEPC range in size from 105-110 kDa (Izui et al., 2004). The C-terminal tetrapeptide in bacterial type PEPC is (R/K)NTG while plant type PEPCs are QNTG (Sanchez and Cejudo, 2003). These C-terminal tetrapeptides can be seen (labeled as I) in AtPPC1, AtPPC2 and AtPPC3 isoenzymes (Figure 1-3). All bacterial type PEPC contains a 10 kDa disordered region (Gennidakis et al., 2007). Most importantly, bacterial type PEPCs lack the N-terminal serine residue required for PPCK phosphorylation (Mamedov et al., 2005). This phosphorylation site is the major structural difference that sets apart plant and bacterial PEPC enzymes.

There are two distinct PEPC classes that were first discovered in the green algae *Selenastrum minutum* (Rivoal et al., 1998) and later in *Chlamydomonas reinhardtii* (Moellering et al., 2007). It was later discovered that the PEPC molecules were made up of both plant type PEPC and bacterial type PEPC subunits. Purified 410 kDa PEPC homotetramers composed solely of 107 kDa plant type PEPC subunits were classified as a Class-1 PEPC. On the other hand, 910 kDa hetero-octamers containing four 118 kDa bacterial type PEPCs and four 107 kDa plant type PEPCs were classified as a Class-2 PEPC. In class 1 PEPC, two of the four p107 subunits are phosphorylated at one time (O'Leary et al., 2011).

Recent research has indicated that plant type PEPC subunits can be post-translationally modified via ubiquitination. While PEPC and PPCK are degraded by polyubiquitination (Agetsuma et al., 2005), monoubiquitination has been shown to play a non-destructive role and could be involved in PEPC regulation. Uhrig et al. (2008) first discovered the monoubiquitination of Class 1 PEPC in developing castor oil seed endosperm.

**Figure 1-3 Multiple sequence alignment of AtPPC1-AtPPC3 isoenzymes.**

Multiple sequence alignment was achieved using ClustalX software.

([www.clustal.org/clustal2](http://www.clustal.org/clustal2)). NCBI genebank accession numbers used were AtPPC1 (AEE32923.1), AtPPC2 (AEC10146.1) and AtPPC3 (AEE75592.1). \* indicates that the amino acid is conserved in all three isoenzymes. “.” indicates that amino acid in the column were strongly conserved ( $>0.5$ ) and “.” indicates that amino acids in the column are weakly conserved ( $\leq 0.5$ ) according to the sequence alignment software. “I” highlights the conserved C-terminal plant type PEPC amino acid sequence.



Immunoblotting experiments showed two bands when incubated with Anti-COS plant type PEPC IgG: one 110 kDa and the typical 107 kDa subunit. A 440 kDa PEPC heterotetramer was purified, showing that two of the p107 subunits were ubiquitinated at Lys residue 628. More research is required to fully understand the specific role of ubiquitination in PEPC regulation.

## 1.7 Characterizing the PEPC-Cd relationship using the Arabidopsis model system

Using a mutagenetic approach, Arabidopsis has been used to identify genes involved in plant carbohydrate metabolism during nutrient stress (Gregory et al., 2009) and the uptake and sequestration of toxic metals (Besson-Bard et al., 2009). More specifically, scientists have identified genes that regulate PEPC and PPCK isoenzyme production (Sanchez and Cejudo, 2003; Lebouteiller et al., 2007; Gregory et al., 2009).

On the genetic level, PEPC production is controlled by four genes in Arabidopsis; *AtPPC1* – *AtPPC4*, with the first three coding for Class 1 PEPCs and the fourth contributing to Class 2 PEPC (Sanchez and Cejudo, 2003). The four genes differ in amino acid sequence as well as in their enzymatic structure (Fig. 1.3). *AtPPC1* – *AtPPC3* have high similarity in amino acid sequence, ranging from 84% to 91% (Sanchez and Cejudo, 2003). In contrast, *AtPPC4* has a very low level of amino acid similarity to *AtPPC1* – *AtPPC3*, ranging from 39% to 40% but a slightly higher similarity, 42%, to EcPEPC in *Escherichia coli* (Sanchez and Cejudo, 2003).

Research on PEPC has only recently shifted from its role in photosynthesis to its role in alleviating metal toxicity and essential element deficiency. As previously mentioned, Begum et al. (2009) looked at the effect of Al on transgenic rice and found that C<sub>4</sub> plants that over-expressed PEPC were more tolerant to Al and had increased amounts of organic acids in both the roots and leaves. Gregory et al., (2009) showed that P<sub>i</sub> deficiency induced an up-regulation of *AtPPC1*, *AtPPCK1* and *AtPPCK2* in Arabidopsis, which is also suggestive of a relationship between the requirement for more organic acids and PEPC activity. Given the documented and predictable responses of Arabidopsis to metal

toxicity and nutrient deficiency, the use of this plant is ideal for investigations of the relative roles of PEPC and PPCK production on mitigating Cd-induced stress.

Compared to determining the functional role of PEPC, few studies have characterized the role each PEPC isoenzyme has in plant biochemistry. Under normal growing conditions *AtPPC1* (and its *OsPPC1* equivalent) transcripts were found in high abundance across all plant organs in Arabidopsis and rice; *AtPPC3* had greater transcript abundance in roots and *AtPPC2* transcripts were more abundant in green tissues (Sanchez and Cejudo, 2003). The expression of these genes varies in response to nutrient deficiency. For example, P<sub>i</sub>-deficient Arabidopsis showed 2-fold up-regulation of *AtPPC1* and slight up-regulation of *AtPPC2* in both shoots and roots (Gregory et al., 2009). Since the PEPC pathway produces metal-chelating intermediates, plants lacking one of the PEPC isoenzymes might be less tolerant to a Cd-induced stress. On the other hand, if PEPC is involved in a general plant-mediated response to metal toxicity then exposing Arabidopsis to a Cd-rich environment will result in up-regulation of each of *AtPPC1* - *AtPPC3*.

A few lines of Arabidopsis with a mutation related to PEPC have been created.

Lebouteiller et al. (2007) inserted *Sorghum* C<sub>4</sub> PEPC T-DNA into Arabidopsis and created two plant lines with either a 2- or 4- fold increase in PEPC activity. These over-expressing lines of C<sub>4</sub> PEPC had a significant increase in PEPC activity in seed tissues but a decrease in PEPC activity as well as expression of *AtPPC1* and *AtPPC2* in leaf tissue in comparison to wild type plants (Lebouteiller et al., 2007).

To study the effects of native PEPC isoenzymes under any type of stress in a C<sub>3</sub> plant such as Arabidopsis, mutant lines in which one of the PEPC isoenzymes is knocked out are required. Arabidopsis provides a good model for studying the effects of Cd toxicity on the PEPC pathway because mutants that lack the genes for *AtPPC1-AtPPC3* are readily available. For the purposes of this project, three T-DNA insert knockout mutant lines were procured from the Arabidopsis Biological Resource Center, each line has a corresponding knockout for one of the three class 1 PEPC isoenzymes (Alonso et al., 2003). Gene knockouts will be referred to as *Atppc1* – *Atppc3*. Their SALK ascension

codes are SALK\_070605C (*Atppc1*), SALK\_128516C (*Atppc2*) and SALK\_031519C (*Atppc3*).

## 1.8 Thesis objectives and hypothesis

Improvements in our understanding of plant-metal uptake and sequestration, from single-pathway biochemistry to the environment at large, is essential for understanding factors that affect the efficiency of both phytoremediation processes and the use of bioengineering for agricultural purposes. Advancements in this field could help us to understand how specific metals are taken up and stored in the plant, and make improvements for healthier, less contaminated crops.

It has been generally accepted that plants have some control over the uptake and sequestration of metals, both essential and toxic, but the specifics of these processes are not well understood. Given the importance of organic acids to ameliorating metal toxicity and nutrient deficiency, and the importance of PEPC on the production of organic acids it is expected that the presence of Cd in nutrient media will induce an up-regulation of PEPC and increased production of organic acids.

The study presented in this thesis was designed to investigate the relationship between the production of TCA cycle intermediates and Cd stress using *Arabidopsis* knockout lines in which the *AtPPC1-AtPPC3* genes have been disabled, as well as a wild-type line. The specific objectives were:

1. To determine if the absence of one of the three PEPC isoenzymes affects seedling development under Cd-induced stress by measuring biomass, rosette diameter, chlorophyll content and chlorophyll fluorescence.
2. To determine the changes in the transcript abundance for each isoenzyme, the PEPC protein abundance and enzymatic activity of PEPC as well as the predominant organic acids produced in both root and shoot tissues when plants experience Cd stress.

It is hypothesized that when *Arabidopsis* seedlings are exposed to  $\text{Cd}^{2+}$  there will be an increase in Class 1 PEPC and PPCK relative transcript abundance, relative protein abundance, PEPC protein phosphorylation and activity level of both PPCK and PEPC. Furthermore, there will be an increase in total organic acid concentration in both shoot and root tissues.

In order to measure the role of Class 1 PEPCs in Cd-induced stress, *Arabidopsis* seedlings must be grown in conditions that lack other potential environmental stresses. To accomplish this, seedlings will be grown under aseptic conditions in hydroponic cultures. This will remove the chance of colonization by microbial communities, which could mask the effects of Cd-induced stress in plants by either forming a mutualistic relationship with the plants roots or compounding the deleterious effects of metal toxicity. Nutrients, as well as  $\text{Cd}^{2+}$ , are more readily available in hydroponic media than soil thus eliminating the possibility of nutrient deficiency; a soil matrix could reduce metal solubility and uptake. Hydroponic culture also results in larger root biomass of *Arabidopsis* as well as minimizes root loss and damage during plant harvest (Hétu et al., 2005).

Modern molecular methods can be used to determine whether or not the PEPC pathway is involved with the plant's response to Cd-induced stress. To get an accurate picture of what is happening, all of the stages from transcript abundance to enzyme activity must be investigated. One molecular technique for rapid analysis of relative transcript abundance using quantitative reverse transcription PCR was modified by Pfaffl (2001). Relative quantification is more applicable to this project than is absolute quantification because it allows comparison of changes in transcript abundance among treatments. While it does not give an absolute number of the transcripts present in a sample, relative quantification does allow for a more accurate comparison among multiple tissue and treatment types (Simon, 2003). Variability among samples can be masked by analytical variability related to RNA extraction, purification, efficiency, and/or pipetting error. Therefore, care must be taken at all steps of analysis to reduce any chance of error (Wong and Medrano, 2005; Kubista et al., 2006).



It is important to measure whether or not gene transcript abundance effectively translates into increased protein abundance as well as whether or not said protein is functional (e.g., phosphorylated). This can be measured for PEPC with the use of SDS-PAGE and immunoblotting. Specific antibodies that have been successfully used with Arabidopsis in previous studies (Gregory et al., 2009) can be used to determine the relative abundance of PEPC present in each sample as well as the level of PEPC-specific phosphorylation. Immunoblotting does have some limitations. The antibodies used in this study are unable to distinguish between PEPC isoenzymes. This flaw can be overcome with the use of PEPC gene knockout lines to measure how Arabidopsis seedlings react to the absence of one of the three Class 1 PEPCs. Accurate repeatability can also be an issue with immunoblotting; therefore, a known concentration of a standard peptide must be run with each blot. This will eliminate any discrepancies in terms of blot exposure time, variation in antibody concentration or buffer concentrations. Most of the limitations discussed for RT-qPCR and immunoblotting can be overcome with consistent and careful technique.

## 2 Methods

### 2.1 Plant material and growth conditions

*Arabidopsis* (Col-0) *Atppc1*, *Atppc2* and *Atppc3* seeds were obtained from the *Arabidopsis* Biological Resource Center. Each knockout line contains a T-DNA insertion in the region coding for one of the plant-type PEPCs and all *Arabidopsis* used to produce bulk seed stocks by the *Arabidopsis* Biological Resource center were analyzed by PCR to confirm the homozygosity of each line (Alonso et al., 2003). In order to maintain homozygosity of the seed stock, *Arabidopsis* plants were grown in pots surrounded by cellophane cones to minimize the potential for cross-pollination. Lines were also tested with PCR to verify homozygosity. A wild type seed stock was generated from seeds donated by the Krishna lab (Western).

Seedlings were grown hydroponically, using a technique modified from Héту et al. (2005). All transfers were done using aseptic techniques in a laminar flow hood. Seeds were surface-sterilized in a microcentrifuge tube with 1 mL of 70% ethanol for 5 min, after decanting the ethanol they were rinsed in 30% bleach solution for 8 min, and then rinsed three times with autoclaved reverse osmosis (RO) water. A small volume of autoclaved RO water was left in the microcentrifuge tube to avoid seed desiccation. Seeds were stored at 4°C in the dark for three days to synchronize germination.

For the rosette diameter (section 2.2.2), chlorophyll fluorescence (section 2.2.4), and chlorophyll content (section 2.2.5) measurements, seeds were transferred directly onto approximately 20 mL of 0.5× Murashige and Skoog (MS) medium (with 1% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8) in sterile Petri plates (Appendix A).

For the Cd dose-response study (section 2.2.1) and determination of Cd concentration (section 2.2.3) as well as investigations of the transcript abundance, activity and phosphorylation of PEPC and PPCK, and production of organic acids (section 2.3), the seeds were germinated on nutrient agar then transferred to liquid growth medium as follows. Before seed transfer, sterile 2.5 cm<sup>2</sup> pieces of fiberglass screen (Easy Screen,

RRC International Inc., Quebec) were placed on approximately 20 mL of 0.5× MS medium (with 1% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8) in sterile Petri plates using ethanol-flamed forceps. Mesh screens acted as a support matrix for the roots and minimized root damage during transfer to liquid culture. Batches of 15 seeds were pipetted onto mesh screens, the Petri plates were sealed with Parafilm, then placed in a growth chamber with a 16:8 hour light: dark cycle maintained at 60% RH and 22°C. Light intensity at the seedling level was determined to be  $185 \pm 5.5 \mu\text{mol}/\text{m}^2/\text{s}$  using a Li-250 light meter (LiCor, Nebraska).

After seven days, seedlings growing on the mesh screens were transferred using ethanol-flamed forceps into sterile jars containing 10 mL of 0.5× MS medium (with 1% (w/v) sucrose, 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) under 16 h day conditions ( $113 \pm 5.7 \mu\text{mol}/\text{m}^2/\text{s}$  at the seedling level due to light attenuation through the glass jar) at 60% RH and 22°C. After seven days, plants were transferred to fresh 20 mL media containing one of the experimental Cd concentrations. Petri plates and jars were rotated daily to minimize position-dependent variation in growth chamber conditions. At 21 days in the growth chamber, plants were harvested.

## 2.2 Measures of Cd stress

### 2.2.1 Biomass

A preliminary dose response study was used to determine the optimal Cd concentrations for testing the Cd-induced stress responses of PEPC isoenzymes. The experimental treatments included a range (0, 0.1, 1.0, 5.0, 7.5, or 10  $\mu\text{M}$ ) of  $\text{CdCl}_2$  concentrations in the nutrient agar, with four replicates per treatment. Wild type and *Atppc1 – Atppc3* were grown as described in section 2.1 for 21 days and the number of seeds per plate was recorded. Upon harvest, root and shoot tissues were separated, fresh weight (FW) was recorded, and tissue was placed in pre-dried, pre-weighed Al foil envelopes. Sealed

envelopes were oven-dried at 60°C to constant weight (approximately 7 days). The root and shoot dry weight (DW) was recorded.

Concentrations of Cd chosen for further study were among those that induced no more than a 30% reduction in dry weight relative to control; for accurate assessment of the physiological responses to Cd, the plants should be Cd-stressed but not experiencing Cd toxicity.

### 2.2.2 Rosette diameter

Plants were grown on agar plates as was described in section 2.1 with CdCl<sub>2</sub> (0, 0.1, 1.0, 5.0, 7.5, or 10 µM) for a total of 14 days. Five seeds were pipetted onto each plate, 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 (Rasband, 2012). To calibrate the software, rosette diameters were also measured using digital calipers.

### 2.2.3 Cd concentration

To measure Cd concentration in plants, seedlings were grown as described in section 2.1, with three CdCl<sub>2</sub> treatments (0, 1, 5 µM) as determined by the dose response study. Root and shoot tissues were separated and oven-dried to constant weight as described in section 2.2.1. Dried tissue was ground into a fine powder using a mortar and pestle. Powdered sample (0.050 g shoot or 0.025 g root tissue) was placed in a 15 mL acid-washed test tube. Dried, ground tomato leaves (NIST standard reference material #1573a) and reagent blanks were also assayed for Cd concentration to determine percentage recovery of Cd in tissue samples and to test for contamination, respectively.

All test tubes were placed in a rack and shoots and tomato leaves were digested in 500 µL nitric acid (OmniTrace<sup>®</sup>, EM Science, USA); roots were digested in 250 µL nitric acid. Each test tube was covered with an acid-washed marble in order to prevent evaporation of sample while allowing for the release of pressure within the tube. Samples remained in the fume hood overnight, which allowed the digestion to proceed at a slow rate. On

the following day, the test tubes and rack were transferred into a tray filled with 3 cm of fine sand. The entire tray was transferred onto a hot plate and heated at 90°C until the vapors were transparent. Sand helped to ensure an even distribution of heat amongst the test tubes. Once cooled, samples were filtered (Whatman 403 grade paper) and diluted with RO water to 10 mL for shoot, and 5 mL for root, samples. Samples were analyzed for Cd concentration using inductively-coupled plasma atomic emission spectrometry (ICP-AES) following Adeniji et al., (2010). The final concentration of nitric acid in each diluted sample was below 5% (v/v) to avoid interference with ICP-AES measurements.

#### 2.2.4 Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was measured in both mutant and wild-type seedlings grown at two light conditions (105 and 185  $\mu\text{mol}/\text{m}^2/\text{s}$ ) to (1) determine whether there was a difference in PS II photochemical efficiency between the two light intensities used in the growth protocol (section 2.1) and (2) determine if the absence of one of the PEPC isoenzymes affects PS II efficiency. Seedlings were grown on nutrient agar as described in section 2.1, and subjected to one of the two light treatments as well as one of three  $\text{CdCl}_2$  treatments (0, 1, 5  $\mu\text{M}$ ) chosen from the dose response study. After fourteen days, seedlings were dark adapted for 1 hour at room temperature before PS II efficiency was measured using a PAM chlorophyll fluorometer (Heinz Walz, Germany). Plant shoots were exposed to a single short (800 ms) pulse of saturating blue light ( $\lambda = 470 \text{ nm}$ , 6000  $\mu\text{mol}/\text{m}^2/\text{s}$ ) from the PAM photodiode (IMAGE-L; Heinz Walz, Germany). The maximum photochemical efficiency of PSII was calculated as  $F_v/F_m$  (Maxwell and Johnson, 2000).

#### 2.2.5 Chlorophyll content

The chlorophyll content of shoot tissue was determined using a protocol modified from Pockock et al., (2004). Shoot tissue from experiment 2.2.4 was excised and 0.1 g was frozen in liquid nitrogen and ground into a fine powder. Cold samples were homogenized in 1 mL chlorophyll extraction buffer (80% (v/v) aqueous acetone, 2.5 mM sodium phosphate, pH 7.8) to minimize the conversion of chlorophyll into phaeophytins. The

samples were then centrifuged for 5 min at 3024 g at 4°C to remove whole chloroplasts. A 0.05 mL aliquot of pigment extract was diluted in 0.95 mL of chlorophyll extraction buffer. Samples were vortexed for 15 s and 200 µL of each 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, CA, USA). Chlorophyll content and the chlorophyll a:b ratio were calculated using equations from Porra et al., (1989).

## 2.3 Cd-induced effect on the PEPC pathway

### 2.3.1 RNA extraction and quantification

To quantify PEPC and PPCK isoenzyme gene transcript abundance, *Arabidopsis* wild type and PEPC mutant lines were grown as described in section 2.1 in medium containing 0, 1, or 5 µM CdCl<sub>2</sub> with three replicates per treatment. The 1 and 5 µM CdCl<sub>2</sub> concentrations were chosen based upon the dose response study (rosette and biomass measurements) as well as visual observations. Symptoms of Cd-stress (chlorosis) were noted in seedlings grown at 10 µM and to a lesser extent 7.5 µM CdCl<sub>2</sub>. Seedlings grown at 0.1 µM CdCl<sub>2</sub> were not significantly different from those grown without cadmium. Only *Arabidopsis* grown in 1 µM and 5 µM CdCl<sub>2</sub> concentrations had a reduction in size and didn't display leaf chlorosis.

Shoot and root tissues were separated and flash-frozen in liquid nitrogen and RNA was extracted using a modified Trizol method (Chomczynski and Sacchi, 1987). A 0.2 g subsample was ground to a powder and transferred to a chilled 1.5 mL microcentrifuge tube. Tissue was homogenized with 0.5 mL Trizol Reagent (Invitrogen) and vortexed for 3 min before being incubated at room temperature for 5 min. Samples were homogenized a second time with 0.2 mL chloroform and vortexed for 1 min before being centrifuged for 15 min at 11,500 rpm. Supernatant was transferred into a clean microcentrifuge tube containing 0.5 mL isopropanol and briefly vortexed before incubation at room temperature for 15 min and centrifugation (4°C) for 20 min at 11,500 rpm. The supernatant was discarded and the pellet was washed with 1 mL 75% (v/v) ethanol, vortexed, and then centrifuged (4°C) for 15 min at 9000 rpm. Supernatant was

syphoned off and the pellet was dried for 10 min before being suspended in diethylpyrocarbonate (DEPC)-treated RO water.  $A_{260}/A_{280}$  absorbance measurements of each sample were taken to determine RNA concentration (Veljanovski et al., 2006) and then subsamples were run on an agarose gel to assess RNA integrity.

### 2.3.2 Quantitative RT-PCR of PEPC and PPCK genes

Relative transcript abundance was quantified using the method of Pfaffl (2001). Samples were treated with DNase I (Invitrogen) to remove contaminating genomic DNA (1  $\mu$ g RNA sample, 1  $\mu$ L 10 $\times$ DNase I reaction buffer, 1  $\mu$ L DNase I) in a total volume of 25  $\mu$ L DEPC-treated water and incubated at 37°C for 30 min. cDNA was synthesized by placing all components on ice and then mixing 4  $\mu$ L 5 $\times$  qScript cDNA SuperMix (Quanta Biosciences) and 1  $\mu$ g RNA template in a total volume of 20  $\mu$ L using DEPC-treated water in a 0.2 mL microcentrifuge tube. Microcentrifuge tubes were vortexed and then briefly centrifuged to collect components at the bottom of the reaction tube. Samples were immediately incubated in a Bio-Rad CFX96 Real Time PCR detection system at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min. Samples were then stored at -20°C until use. qPCR was performed according to the instructions provided for the Bio-Rad CFX96 Real Time PCR detection system. In each 96-plate well the following components were added: 10  $\mu$ L of 2 $\times$  PerfeCTa SYBR Green FastMix (Quanta Biosciences), 5  $\mu$ L cDNA template, 2  $\mu$ L each of reverse and forward primer of interest topped to 20  $\mu$ L with DEPC-treated water. 96 well plate was sealed and then loaded in into the PCR detection system, with the following cycling protocol: one cycle (95C 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 (Appendix B) were used to amplify Arabidopsis *PEPCs* (*AtPPC1*, *AtPPC2*, and *AtPPC3*) and *PPCKs* (*AtPPCK1* and *AtPPCK2*); *UBQ10* was used as the reference gene. Relative transcript abundance was calculated using Pfaffl's (2001) method. Primer efficiency was tested beforehand by running a standard curve in triplicate. Efficiency was calculated with the following formulae (Pfaffl, 2001):

$$\text{Efficiency} = 10 \left( -\frac{1}{\text{slope}} \right)$$

$$100\% \text{ Efficiency} = 10 \left( -\frac{1}{-3.321928} \right) = 2$$

The cycle threshold (Ct) is the number of cycles required for the fluorescence signal to exceed background fluorescence levels. The slope of the standard curve (Ct versus concentration of sample used) is related to the efficiency of the PCR reaction. Acceptable primer efficiency was defined as within 10% of exact doubling for each cycle and within 5% of each gene-specific primer set used. Relative transcript abundance was calculated with the following formula;

$$\text{Ratio} = \frac{(\text{Efficiency of target gene})^{\Delta\text{Ct}(\text{control}-\text{Cd treatment})}}{(\text{Efficiency of reference gene})^{\Delta\text{Ct}(\text{control}-\text{Cd treatment})}}$$

Relative transcript abundance is a ratio between the efficiency of *AtPPC* or *AtPPCK* primer amplification and the efficiency of the *UBQ10* reference primer amplification. Each primer set compares the control (wild-type shoot 0  $\mu\text{M}$   $\text{CdCl}_2$ ) to the plant lines treated with Cd (specific plant line, specific tissue type, and one of 0, 1, or 5  $\mu\text{M}$   $\text{CdCl}_2$  treatments).

### 2.3.3 PEPC crude protein extracts

Plants roots and shoots of 21-day-old plants were harvested, frozen with liquid nitrogen and then stored at  $-80^\circ\text{C}$  until ready for use. Root and shoot tissues were homogenized separately according to Gregory et al. (2009). One gram of frozen tissue was ground into a powder in a cold mortar and PEPC extraction buffer (100 mM HEPES-KOH pH 8.0, 10 mM EDTA, 2 mM EGTA, 0.2% (v/v) Triton X-100, 1% (w/v) PVPP, 50 mM NaF, 1 mM  $\text{Na}_2\text{VO}_3$ , 1 mM  $\text{Na}_2\text{MoO}_4$ , 2 mM DTT, 1 mM PMSF, 2 mM DPDS) was added (1:2 (w/v) leaves, 1:3 (w/v) roots) and the mixture was ground until it was a liquid slurry. Sample was placed into 1.5 mL tube and immediately centrifuged for 15 min (14,000 rpm at  $4^\circ\text{C}$ ). The supernatant was collected and stored in two clean microcentrifuge tubes at  $-80^\circ\text{C}$ . The total amount of protein was determined using a Bradford assay (500-0006;



Bio-Rad; Bradford, 1976). Absorbance was measured at 595 nm using a Spectramax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Each 96 well plate was standardized using a  $\gamma$ -globulin (Bio-Rad) dilution curve. To remove pipetting error, each sample was run in duplicate and with two volumes (5  $\mu$ L or 10  $\mu$ L).

### 2.3.4 PEPC semi-quantification with immunoblotting

Protein extracts were heated along with a PEPC control sample for 5 min at 90°C and then fractionated using SDS-PAGE according to Tsang et al. (1983) on an equal protein basis (Appendix C; 20  $\mu$ g per lane). Proteins were blotted onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature in 5% (w/v) skim milk TBS buffer (20 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween20, pH 7.6) then washed three times (20 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween20) for 5 min each time. Washed membranes were incubated for 1 h in 1:2000 dilution (v/v) of either rabbit anti-cos p107 IgG to measure plant type PEPC or rabbit anti-cos pSer11 IgG to measure phosphorylation state (supplied by Dr. WG Plaxton, Queens University). 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 enhanced chemiluminescence according to the manufacturer's instructions (ECL; Amersham-Pharmacia Biotech). Bands were quantified using Image J software (Rasband, 2012). Relative phosphorylation was measured by taking the ratio of pSer11 and p107 band intensities.

### 2.3.5 PPCK crude extractions

Roots and shoots of 21-day-old plants were harvested, frozen in liquid nitrogen, and stored at -80°C until use. One gram of frozen tissue was ground into a powder and homogenized in 1:2 (w/v) PPCK extraction buffer (50 mM HEPES-KOH (pH8.0), 1 mM EGTA, 1 mM EDTA, 10 mM MgCl<sub>2</sub>, 5% (w/v) glycerol, 0.01% (v/v) triton X-100, 5 mM thiourea, 1 mM DTT, 2 mM DPDS, 2% (w/v) PVPP) and the mixture was ground until it had the consistency of a liquid slurry. Sample was placed into 1.5 mL tube and immediately centrifuged for 15 min (14,000 rpm at 4°C). The supernatant was collected

and stored in two clean microcentrifuge tubes at  $-80^{\circ}\text{C}$ . The total amount of protein was determined using a Bradford assay (500-0006; Bio-Rad; Bradford, 1976). Absorbance was measured at 595 nm using a SpectramMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Each 96 well plate was standardized using a  $\gamma$ -globulin (Bio-Rad) dilution curve. To remove pipetting error, each sample was run in duplicate and with two volumes (5  $\mu\text{L}$  or 10  $\mu\text{L}$ ).

### 2.3.6 PPCK assay

The activity of PPCK was assayed following the methods of Murmu and Plaxton (2007) in shoot and root extracts from section 2.3.5. A 20  $\mu\text{g}$  sample of crude protein extracts (Appendix C), and 10  $\mu\text{g}$  of purified PEPC (supplied by WC Plaxton), were dephosphorylated in dephosphorylation buffer (50 mM Tris-HCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 20% (v/v) glycerol), 5 U/mL PP2A) and incubated at  $30^{\circ}\text{C}$  for 30 min. Following dephosphorylation, samples were incubated with PPCK assay mix (50 mM HEPES-KOH (pH 8.5), 5 mM  $\text{MgCl}_2$ , 0.2 mM EGTA, 4 mM phosphocreatine, 5 U creatine phosphokinase, 0.25 mM  $\text{P}^1\text{P}^5$ -di(adenosine-5<sup>1</sup>)-pentaphosphate, 1 mM DTT, 50 nM microcystin-LR and 0.1 mM ATP). Addition of ATP initiated the reaction. Samples were incubated at  $30^{\circ}\text{C}$  for 15 min, heated at  $90^{\circ}\text{C}$  for five min and proteins were separated and detected as described in section 2.3.4. During immunoblotting, anti-cos anti-phosphorylation site specific (APS) IgG was substituted as the primary antibody. Band intensity was measured using image J software. Relative rate of phosphorylation was calculated by taking the ratio of APS and p107 band intensities.

### 2.3.7 PEPC activity assay

The activity of PEPC was assayed (Gregory et al., 2009) at  $24^{\circ}\text{C}$  using protein extracts from section 2.3.3. In each well of a 96 well plate, 5  $\mu\text{L}$  sample was added to 20  $\mu\text{L}$  of autoclaved RO water and 175  $\mu\text{L}$  of assay solution (50 mM HEPES-KOH (pH 8.0), 15% (v/v) glycerol, 5 mM  $\text{MgCl}_2$ , 2 mM DTT, 2 mM  $\text{KHCO}_3$ , 2 mM PEP, 0.15 mM NADH and 5 units $\cdot\text{ml}^{-1}$  malate dehydrogenase) for a total of 200  $\mu\text{L}$ . Samples were run in duplicate and with a double concentration (10  $\mu\text{L}$  sample, 15  $\mu\text{L}$  RO water) to assess precision of activity measurements. Absorbance was immediately measured at 340 nm

using a Spectramax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Duplicates were run and 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  $\mu\text{mol}$  of oxaloacetate per min.

### 2.3.8 Extraction of organic acids from tissue

Organic acids were extracted from plant tissues using a method modified from Xu et al. (2006). Plants were grown as described in section 2.1. Upon harvest, root and shoot tissues were separated, frozen with liquid nitrogen and a subsample of frozen tissue was weighed (approximately 0.5 g shoots, 0.25 g roots), then ground in a chilled mortar into a fine powder. Each powdered subsample was transferred into a clean acid-washed test tube. Chilled 0.5 M HCl (3 mL shoots, 1.5 mL roots) was added to the test tubes and they were immediately stoppered with a clean acid-washed marble and transferred to a hot water bath set at 80°C for 20 min. Every 5 min the samples were shaken. Samples were removed from the hot water bath and allowed to cool before being transferred to 15 mL Falcon tubes and diluted to a total volume of 10 mL for shoot samples or 5 mL for root samples. Samples were centrifuged at 2500  $\times$  g for 15 min. The supernatant was collected and filtered (0.45  $\mu\text{m}$ ) into a clean, sterile 15 mL Falcon tube. Samples were stored at 4°C prior to analysis by reverse phase high performance liquid chromatography (RP-HPLC).

### 2.3.9 Organic acid analysis using RP-HPLC

Analysis of organic acids by RP-HPLC (Xu et al., 2006) was performed using an Agilent Infinity 1260 HPLC (Binary Pump, Agilent Technologies, USA). 20  $\mu\text{L}$  of sample was injected onto a Prevail Organic Acid HPLC column (150  $\times$  4.6 mm 5  $\mu\text{M}$ , Alltech, Mandel Scientific Company, Guelph, Ontario) with a mobile phase of 25 mM  $\text{KH}_2\text{PO}_4$ , adjusted to pH 2.5 with phosphoric acid, at a flow rate of 1.0 mL/min. Organic acids were detected at 210 nm with a UV detector. Standards for oxalic, malic, succinic, citric fumaric acids (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 (Appendix D). To verify running conditions, a mixture of standard acids (and a duplicate) was run before each batch of samples. After every twenty samples an HCl and RO water filtration blank were included. Every fifteen 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.

## 2.4 Statistical analysis

SigmaPlot version 11.0 was used for all statistical analyses. Two way analyses of variance (ANOVA) and Tukey's post-hoc tests were performed to detect treatment effects and determine significant differences among means ( $P < 0.05$ ) in mean rosette diameter data. Three way ANOVAs were performed for data from all other experiments in this thesis.

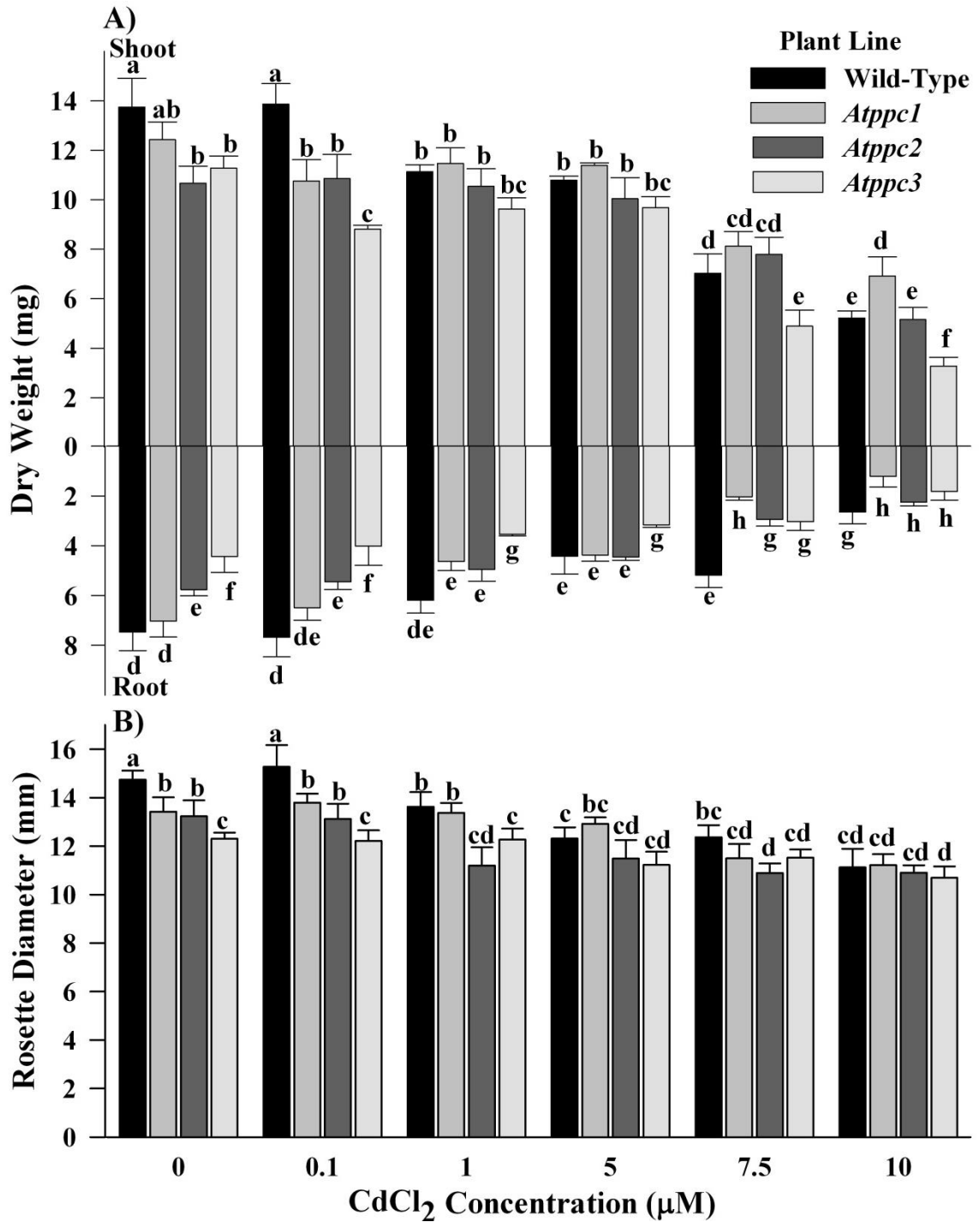
## 3 Results

### 3.1 Effect of Cd and AtPPC knockout on seedling development

Growth measurements of Arabidopsis wild-type and three mutant knockout plant lines grown in a range of CdCl<sub>2</sub> concentrations are shown in Figure 3-1. In all lines, increasing the concentration of CdCl<sub>2</sub> decreased both shoot and root biomass (Figure 3-1A) and rosette diameter (Figure 3-1B). When grown at 10 μM CdCl<sub>2</sub>, all plant lines had an approximately 50% reduction in shoot biomass compared to their respective control plants; rosette diameter was reduced by approximately 20%. Interestingly, under control conditions and at low (0.1 to 5 μM) CdCl<sub>2</sub> concentrations, the mutant lines had up to 40% less shoot and root biomass and up to 15% smaller diameters when compared to the wild type seedlings. The lines in decreasing order of sizes were control > *Atppc1* > *Atppc2* > *Atppc3*. As CdCl<sub>2</sub> concentration increased, the differences among the four plant lines' biomass and rosette diameter were minimal. At 7.5 μM CdCl<sub>2</sub> some plants showed symptoms of Cd-induced stress (chlorosis), which was more predominant in plants grown at 10 μM CdCl<sub>2</sub>.

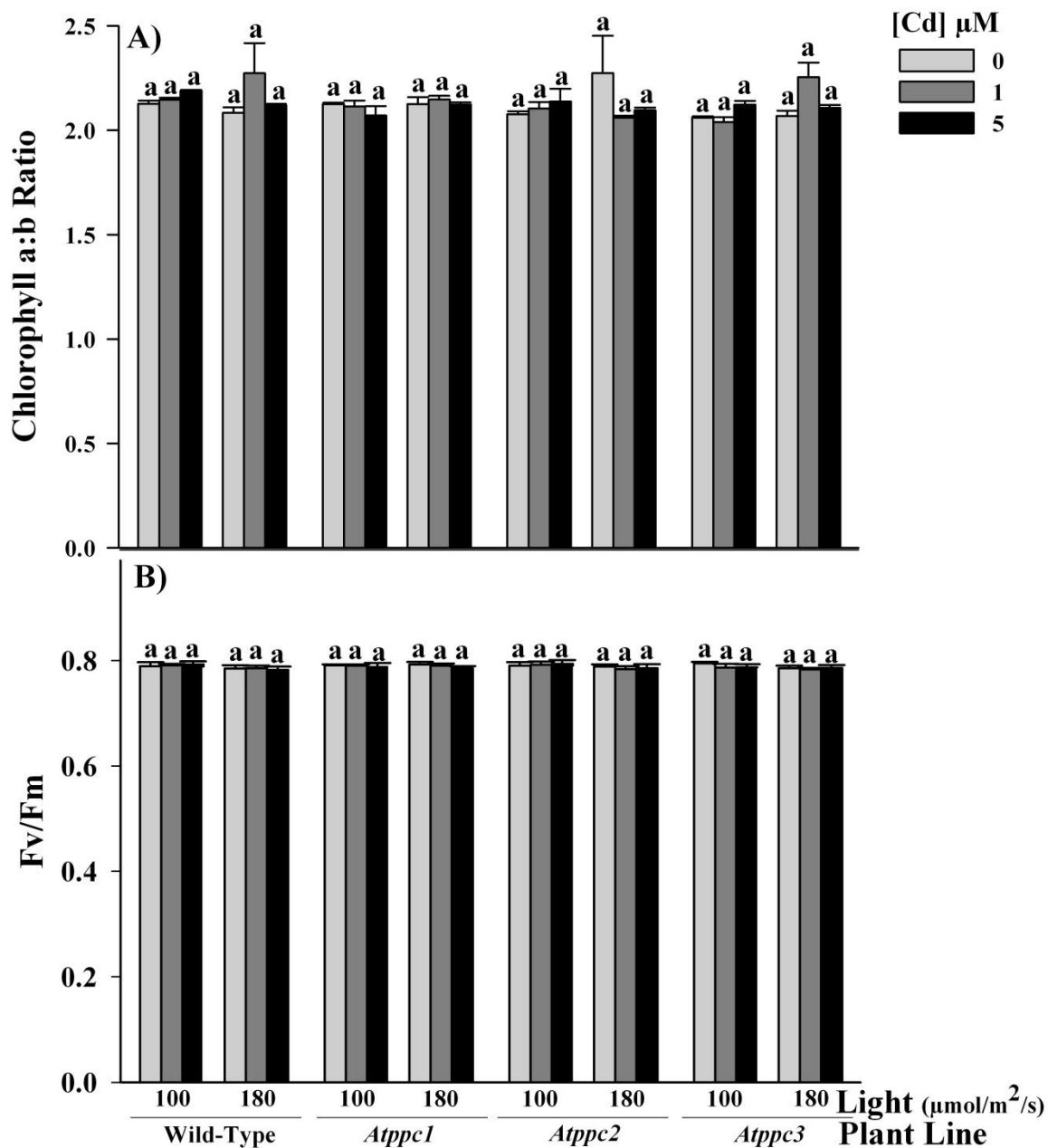
Photosynthetic measurements of Arabidopsis wild-type and mutant plants grown at 0 to 5 μM CdCl<sub>2</sub> concentrations are shown in Figure 3-2. Plants in all treatment types appeared healthy. The chlorophyll a:b ratio was unaffected by CdCl<sub>2</sub> treatment, light intensity or plant line (Figure 3-2A,  $p > 0.05$ ,  $n = 4$ ). Similarly, there was no significant difference in maximum PS II photochemical efficiency among plant lines, CdCl<sub>2</sub> treatments or light concentrations (Figure 3-2B,  $p > 0.05$ ,  $n = 4$ ). All plants in all treatments had an average chlorophyll a:b ratio of  $2.11 \pm 0.04$  and an  $F_v/F_m$  ratio of  $0.788 \pm 0.001$ .

As expected, concentrations of Cd in the tissues of all four plant lines increased as the concentrations of CdCl<sub>2</sub> in the growth medium increased (Figure 3-3). When compared to plants in the 1 μM treatment, there was a 5-fold increase in Cd concentration in plants treated with 5 μM CdCl<sub>2</sub>. In all plant lines, there was a 1.5 to 2-fold higher concentration of Cd in roots than in shoot tissues.



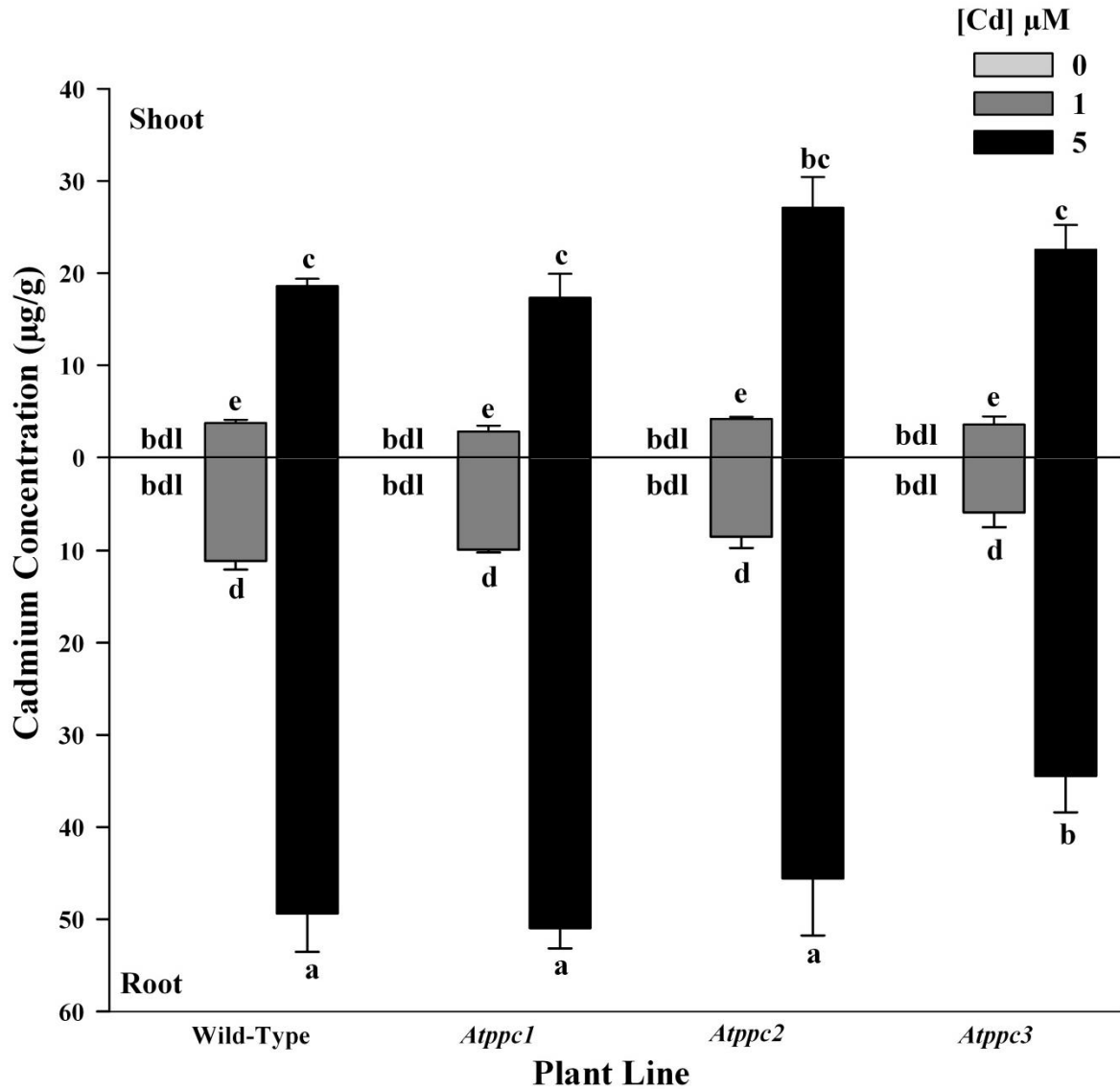
**Figure 3-1 Growth measurements of Arabidopsis at different CdCl<sub>2</sub> treatments.**

Dry weight (A) and rosette diameter (B) measurements of Arabidopsis grown at a range of CdCl<sub>2</sub> concentrations. Vertical error bars represent standard error. Treatments not sharing a common letter are significantly different (3-way ANOVA (A) and 2-way ANOVA (B) with Tukey's post hoc test,  $p < 0.05$ ,  $n = 4$ ).



**Figure 3-2 Photosynthetic measurements of Arabidopsis exposed to increasing concentrations of Cd**

Chlorophyll a:b ratio (A) and photosystem II efficiency (B) measurements in Arabidopsis wild-type and mutant plant lines when grown at 0, 1, or 5  $\mu\text{M}$   $\text{CdCl}_2$  and one of the two (100 or 180  $\mu\text{mol}/\text{m}^2/\text{s}^2$ ) light concentrations used in the growth protocol. Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test,  $p < 0.05$ ,  $n = 4$ ).



**Figure 3-3 Concentration of Cd in shoots (top) and roots (bottom) in Arabidopsis**

Arabidopsis plant lines shoot and root Cd concentration (µg/g) when grown at 0, 1 or 5 µM CdCl<sub>2</sub>. Vertical error bars represent standard error. In some treatments, Cd concentration was below the detection limit (bdl). Treatments not sharing a common letter are significantly different (3-way ANOVA with Tukey's post hoc test,  $p < 0.05$ ,  $n = 3$ ).



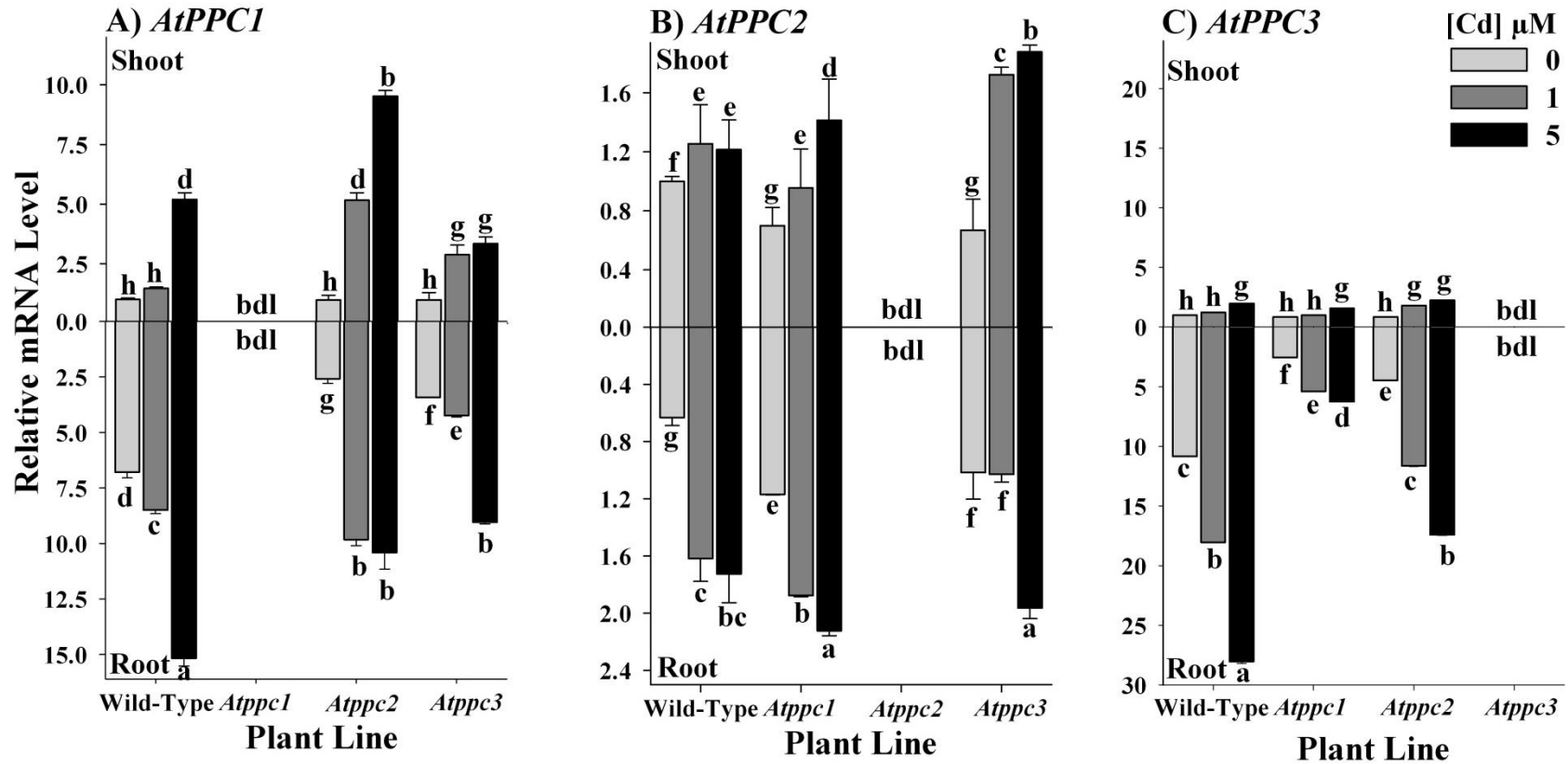
The concentrations of Cd in shoots and roots within each experimental treatment were the same across all four lines; the only exception was a 25-30% lower concentration of Cd in roots of *Atppc3* grown at 10  $\mu\text{M}$  CdCl<sub>2</sub>. Since dry weight and Cd concentration data were collected from independent experiments, correlative statistical analysis was not possible.

### 3.2 Response of PEPC pathway to Cd-induced stress

Analysis of relative transcript abundance in wild-type and knockout mutants when grown in varying CdCl<sub>2</sub> concentrations yielded the predicted results. The relative abundance of transcripts for *AtPPC1–AtPPC3* (Figure 3-4) and *AtPPCK1–AtPPCK2* (Figure 3-5) increased in response to Cd. In addition, the isoenzyme gene knockouts in the mutants were verified because the transcripts of the corresponding isoenzymes were below qPCR detectable limits (Figure 3-4). Furthermore, with all genes tested (*AtPPC1 –AtPPC3*, *AtPPCK1 –AtPPCK2*) transcripts were more abundant in root tissue than in shoot tissue (Figures 3-4 and 3-5).

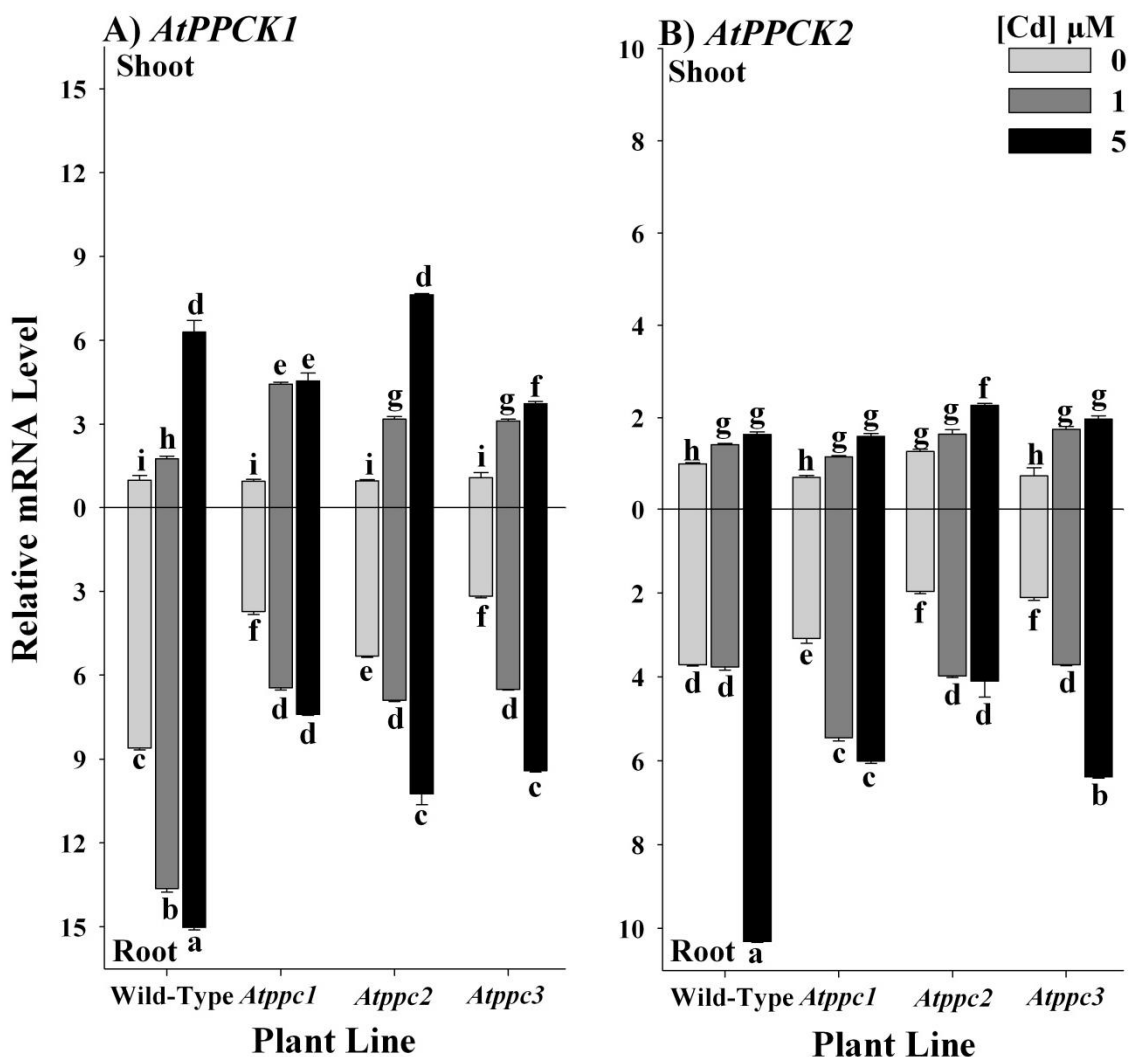
For *AtPPC1*, 1  $\mu\text{M}$  CdCl<sub>2</sub> did not induce increased transcript abundance in shoots or roots of wild-type seedlings but transcript levels increased 5-fold (shoots) and 15-fold (roots) when grown at 5  $\mu\text{M}$  CdCl<sub>2</sub> (Figure 3-4A). In contrast, *AtPPC1* transcript abundance increased in shoots and roots of *Atppc2* and *Atppc3* with 1 or 5  $\mu\text{M}$  CdCl<sub>2</sub>. Surprisingly, in the 5  $\mu\text{M}$  treatment there was more *AtPPC1* transcript in the shoots of the *Atppc2* than in wild-type shoot tissue.

There were no significant changes in *AtPPC2* transcript abundance in shoot tissues of wild-type or *Atppc2* in response to CdCl<sub>2</sub>, but *AtPPC2* transcripts were approximately 2.5-fold higher in Cd-treated shoots of *Atppc3* (Figure 3-4B). In root tissue there was a 1.5-fold relative up-regulation of *AtPPC2* transcripts in all plant lines with the corresponding isoenzyme.



**Figure 3-4 Relative quantification of *AtPPC1* – *AtPPC3* transcript abundance in Arabidopsis**

Relative quantification of *AtPPC1* (A), *AtPPC2* (B), and *AtPPC3* (C) transcript abundance in wild-type and mutant Arabidopsis grown with one of three CdCl<sub>2</sub> treatments (0, 1, 5  $\mu$ M). Levels of mRNA were measured using primers specific for *AtPPC1* (A), *AtPPC2* (B), or *AtPPC3* (C). *UBQ10* was used for a reference gene. Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test, p < 0.05, n = 3).



**Figure 3-5 Relative quantification of *AtPPCK1* and *AtPPCK2* transcript abundance in Arabidopsis**

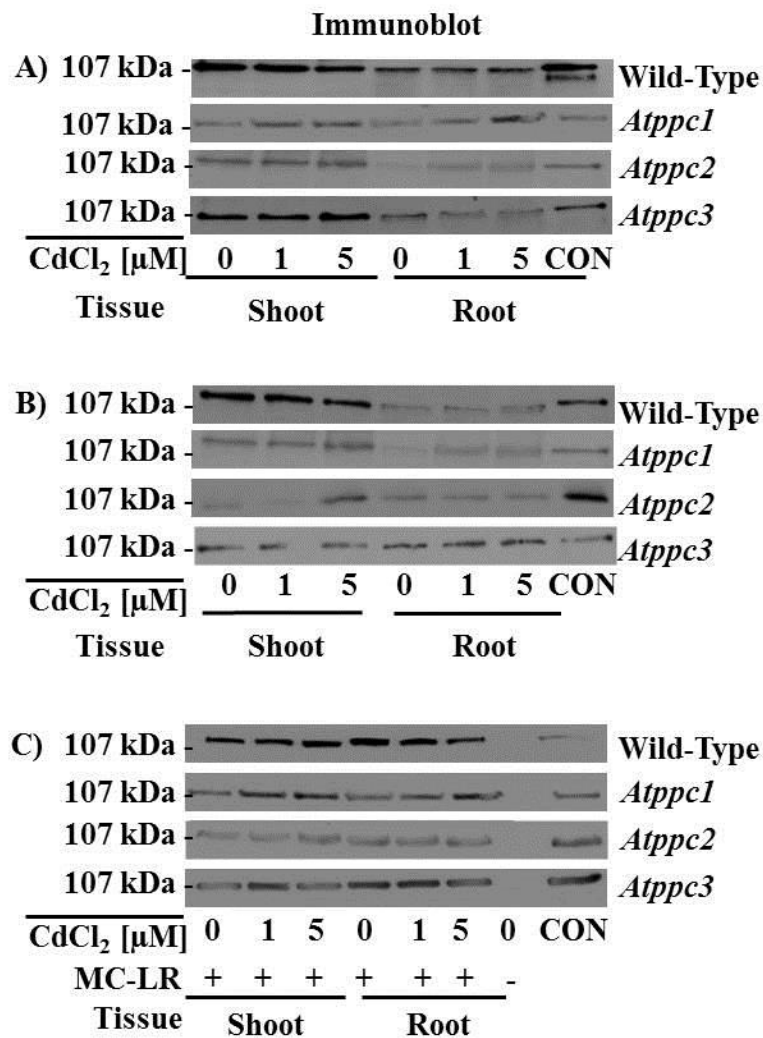
Relative quantification of *AtPPCK1* (A) and *AtPPCK2* (B) transcript abundance in wild-type and mutant Arabidopsis grown with one of three  $\text{CdCl}_2$  treatments (0, 1, 5  $\mu\text{M}$ ). Levels of mRNA were measured using primers specific for *AtPPCK1*. *UBQ10* was used for a reference gene. Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test,  $p < 0.05$ ,  $n = 3$ ).

The relative abundance of *AtPPC3* transcripts followed a pattern similar to that of *AtPPC1*; the relative abundance increased 2- to 3-fold in both shoots and roots in response to 1 or 5  $\mu\text{M}$   $\text{CdCl}_2$  (Figure 3-4C). For this gene, transcript abundance in roots was higher in wild-type plants than in the mutant lines. It should also be noted that there was as much as 20-fold more relative *AtPPC3* in wild-type roots compared to shoots.

The results for *AtPPCK1* and *AtPPCK2* were similar to those of the *AtPPC* genes. There was a 2- and 3-fold up-regulation of *AtPPCK1* and *AtPPCK2* relative transcript abundance, respectively, in response to  $\text{CdCl}_2$  and relative transcript abundance was higher in roots than in shoots (Figure 3-5).

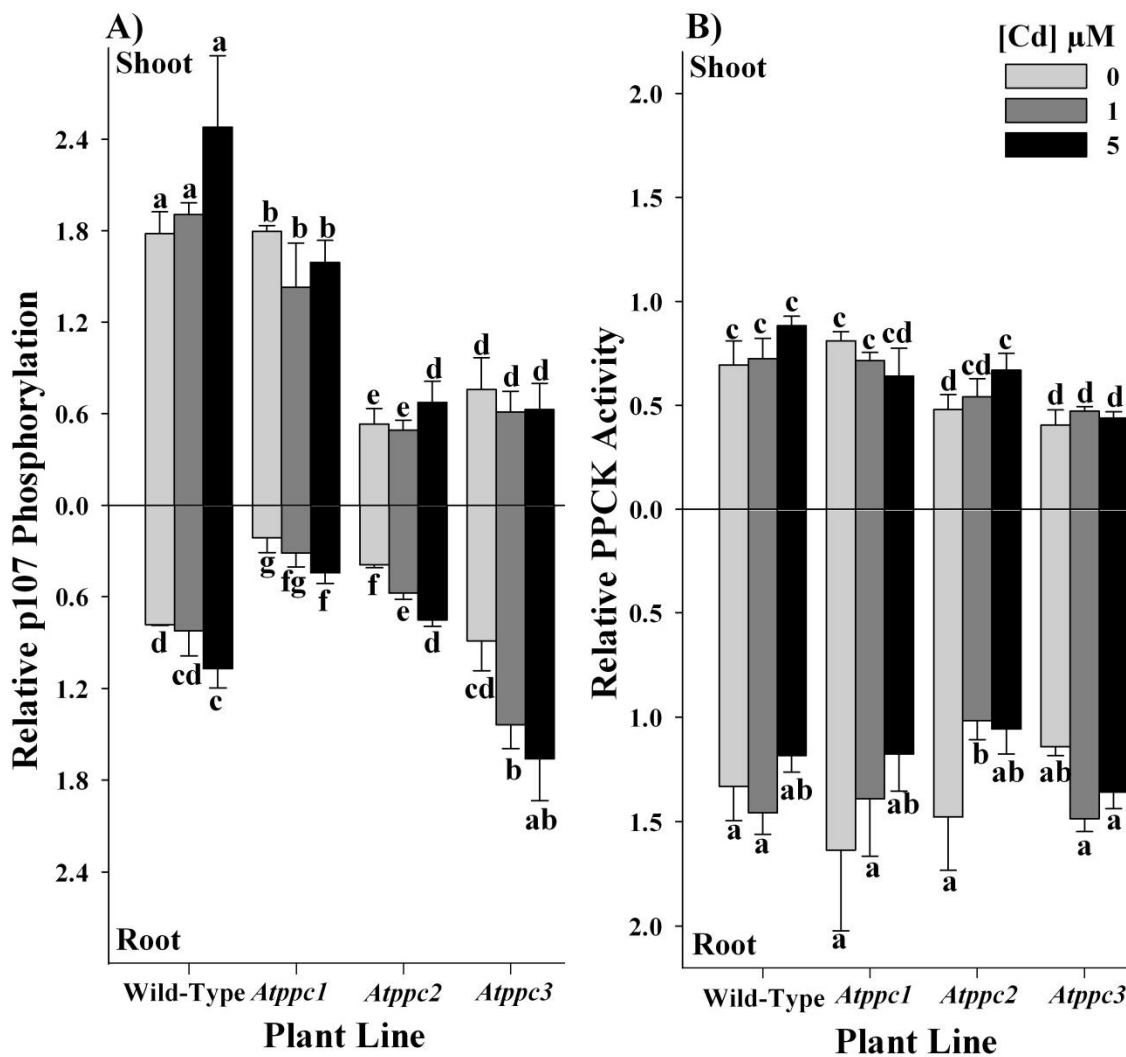
Class-1 PEPC was found in all plant lines and at all  $\text{CdCl}_2$  concentrations in both shoot and root tissues, to varying degrees (Figure 3-6). Furthermore, the PEPC was phosphorylated in all plant lines and  $\text{CdCl}_2$  treatments in both shoot and root tissues. Of the four plant lines, there was more relative phosphorylation in the shoot than in root tissues of wild-type, *Atppc1* and *Atppc2* lines; in contrast, *Atppc3* mutants had significantly more relative phosphorylation in their roots than their shoots (Figure 3-7A). In shoot tissue relative phosphorylation was higher in wild-type than in *Atppc1*, *Atppc2* and *Atppc3*. Surprisingly, there was no significant difference in relative phosphorylation in shoot tissue among  $\text{CdCl}_2$  treatments within plant lines. In root tissue, relative phosphorylation was highest in wild-type and *Atppc3* followed by *Atppc2* then *Atppc1* mutant lines. In root tissue relative phosphorylation increased with  $\text{CdCl}_2$  treatment in all plant lines.

With the exception of the *Atppc2* mutant, the relative PPCK activity did not vary among plant lines or with  $\text{CdCl}_2$  treatment in either shoot or root tissues, although values for roots were 2-fold higher than for shoots (Figure 3-7B). In *Atppc2* shoot tissue, the relative rate of phosphorylation increased by 25% in response to  $\text{CdCl}_2$  whereas it decreased by 25% in roots.



**Figure 3-6 Immunoblots of IgG p107, IgG pSer-11 and IgG APS in Cd treated Arabidopsis**

Immunoblots of p107 (A), pSer 11 (B) and APS (C) of Arabidopsis wild-type and mutant plants when grown with 0, 1, or 5  $\mu$ M CdCl<sub>2</sub>. Abbreviations include anti-phosphorylation site specific antibodies (APS), microcystin-LR (MC-LR), and PEPC protein sample used as a control band (CON).



**Figure 3-7 Relative phosphorylation of class-1 PEPC and PPCK activity in Cd-treated Arabidopsis**

Relative phosphorylation class-1 PEPC (A) and relative PPCK activity (B) of shoots (top) and roots (bottom) in Arabidopsis wild-type and mutant plant lines when treated with 0, 1, or 5  $\mu\text{M}$   $\text{CdCl}_2$ . Relative phosphorylation is the ratio between IgG-pSer11 and IgG-p107 and relative PPCK activity is the ratio between IgG-APS and IgG-p107 as measured on 3 replicate gels similar to that in Figure 3-6. Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test,  $p < 0.05$ ,  $n = 3$ ).

The specific activity of PEPC extracted from shoots and roots of plants grown in a range of CdCl<sub>2</sub> increased 1.5- to 2-fold with increasing CdCl<sub>2</sub> in solution (Figure 3-8). When comparing tissue types, there was 2-fold more PEPC specific activity in root tissue than in shoot tissues. Surprisingly, both *Atppc2* and *Atppc3* had significantly more PEPC specific activity in shoot tissues than both the wild-type and *Atppc1* plant lines in the 5 μM CdCl<sub>2</sub> treatments. Wild-type roots and *Atppc3* had more specific PEPC activity than did *Atppc1* and *Atppc2*.

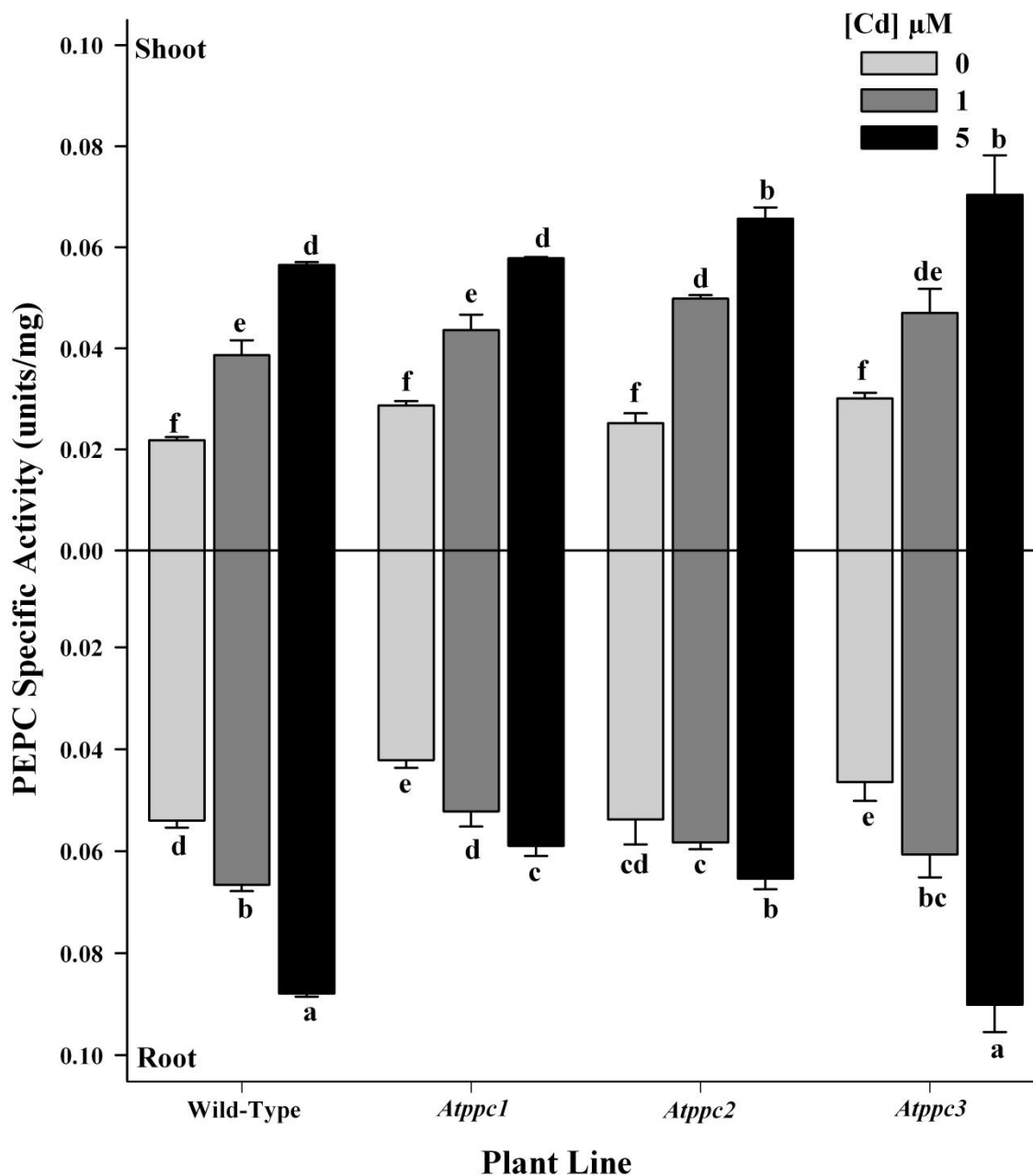
Of the five organic acids measured, oxaloacetic acid had the highest concentrations in both shoot and root tissues (Figure 3-9). The concentration of oxaloacetic acid in shoots varied between 20 and 35 mg/g fresh weight. The concentration of oxaloacetic acid in roots varied between 10 and 15 mg/g fresh weight (Figure 3-9A). Among plant lines, the *Atppc3* mutant had higher concentrations of oxaloacetic acid at each CdCl<sub>2</sub> treatment. The concentration of oxaloacetic acid in shoot tissue increased by 10-15% in all four plant lines as the concentration of CdCl<sub>2</sub> increased. Within roots, the concentration of oxaloacetic acid increased by less than 5% in response to CdCl<sub>2</sub> in all lines except *Atppc3*.

The next most abundant organic acid was citric acid, for which concentrations were about half those of oxaloacetic acid and varied between 4 and 14 mg/g fresh weight in shoots and between 2 and 9 mg/g fresh weight in roots (Figure 3-9B). Concentrations of citric acid did not vary much among plant lines, although they increased in response to CdCl<sub>2</sub> in wild-type and *Atppc3* roots, as well as in *Atppc2* and *Atppc3* shoots grown in 5 μM CdCl<sub>2</sub>. Malic acid concentrations ranged from below detection limit to 0.45 mg/kg in roots and 0.50 mg/kg in shoots and, overall, did not vary among mutant lines (Figure 3-9C). Only in *Atppc2* and *Atppc3* lines did the concentrations of malic acid increase in response to CdCl<sub>2</sub>.

The concentrations of succinic acid varied between 0.5 to 5 mg/kg (Figure 3-9D). With the exception of the *Atppc3* there was more succinic acid in the shoots than in root samples. In shoots, the *Atppc1* – *Atppc3* from the 1 μM CdCl<sub>2</sub> treatment had more succinic acid than did shoots from either of the 0 or 5 μM treatments. Concentrations of

succinic acid increased in response to CdCl<sub>2</sub> in wild-type and *Atppc2* roots but decreased in *Atppc1*. The concentrations of fumaric acid were below the HPLC detectable limit in all root samples and most shoot samples (Figure 3-9E). Measurable fumaric acid varied between 0.17 and 0.22 mg/g fresh weight in wild-type shoots and in *Atppc2* shoots and the 5 μM CdCl<sub>2</sub> treatment. Overall, concentrations of all organic acids combined were 2-fold higher in shoots than in roots (Figure 3-9F). In shoots, the concentrations of organic acids were highest in *Atppc3* plants and increased by 5-15% in response to CdCl<sub>2</sub> in all plant lines (Figure 3-9F). The concentrations of organic acids were more or less equal across root tissues from all lines in all CdCl<sub>2</sub> treatments.



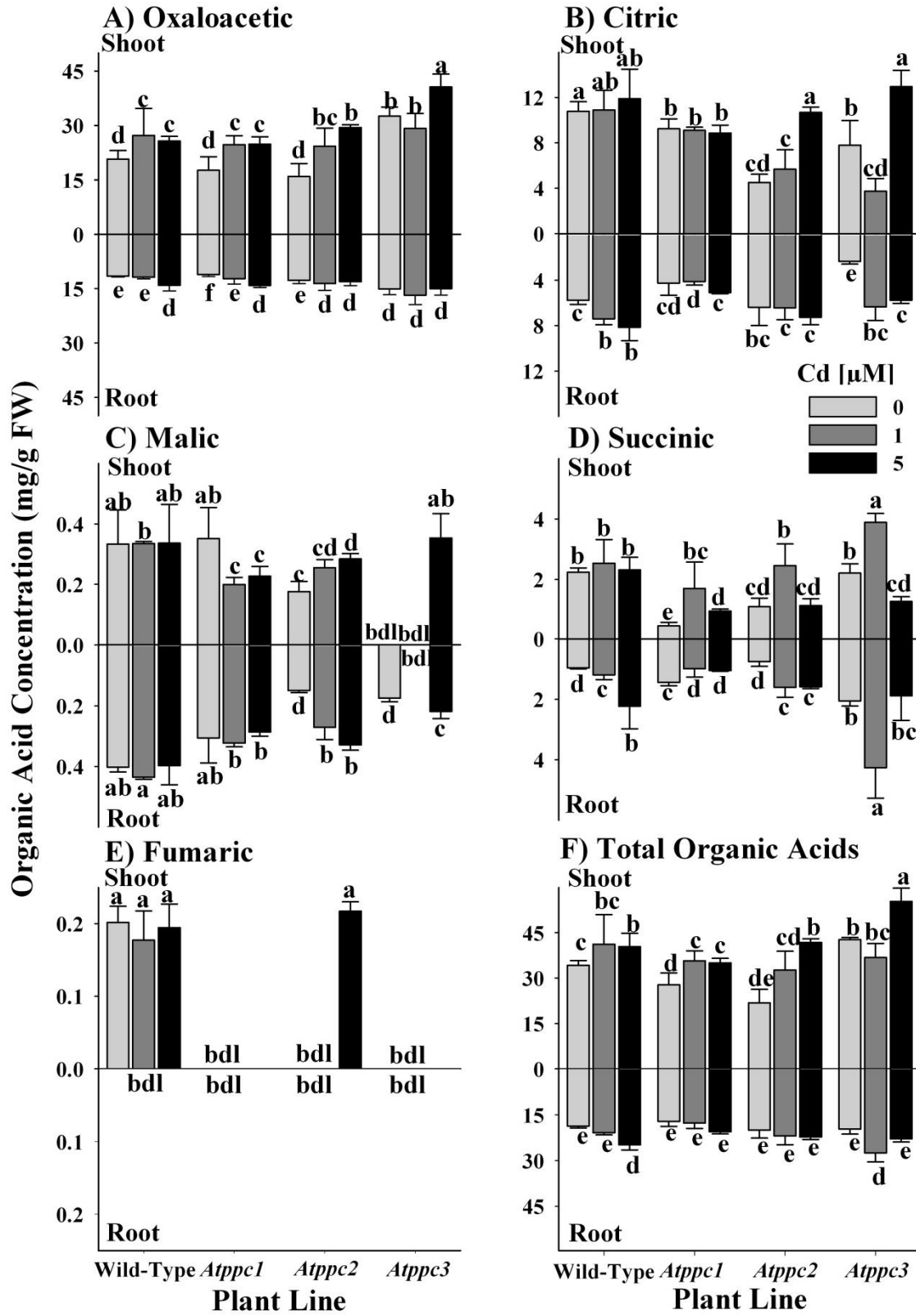


**Figure 3-8 PEPC specific activity of Arabidopsis plant lines exposed to CdCl<sub>2</sub>**

PEPC specific activity in the shoots (top) and root (bottom) of Arabidopsis wild-type and mutant knockout lines exposed to 0, 1, or 5  $\mu\text{M}$  CdCl<sub>2</sub>. One unit of activity is defined as the amount of PEPC resulting from the production of 1  $\mu\text{mol}$  of oxaloacetate per min. Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test,  $p < 0.05$ ,  $n = 3$ ).

**Figure 3-9 Organic acid concentrations in Arabidopsis plant lines exposed to CdCl<sub>2</sub>**

Concentration of oxaloacetic (A), citric (B), malic (C), succinic (D), fumaric acid (E), and the total of A-E (F) in fresh weight (FW) shoots (top) and roots (bottom) tissue from Arabidopsis wild-type and mutant knockout lines grown in either 0, 1, or 5  $\mu\text{M}$  CdCl<sub>2</sub>. Vertical error bars represent standard error. Treatments not sharing a similar letter are significantly different (3-way ANOVA with Tukey post hoc test,  $p < 0.05$ ,  $n = 3$ ).



## 4 Discussion

### 4.1 General overview

My research has improved our understanding of the role of PEPC isoenzymes in Arabidopsis as they relate to Cd stress. Currently, the identification of mechanisms facilitating Cd uptake in plants is a major goal of agricultural bioengineers who aim to produce less contaminated crops for human consumption. I was able to show how the absence of one plant type PEPC isoenzyme in Arabidopsis could affect plant growth as well as the plant's response to Cd.

In 2009, Gregory et al. reported that when Arabidopsis was phosphate starved there was an up-regulation of *AtPPC1* and to a lesser extent *AtPPC2* transcript abundance with high levels of abundance in both roots and shoots. Around the same time PEPC transcript abundance was measured in plants experiencing drought and saline stress (Sánchez et al., 2006). The initial discovery that PEPC isoenzymes displayed differential regulation under other abiotic stresses begged the question: how would the absence of one plant type PEPC alter Arabidopsis' response to Cd?

Research on the differential responses of *AtPPC1* – *AtPPC3* with the use of gene knockout mutants has allowed me to examine the response to Cd of each plant type PEPC in Arabidopsis. This knowledge of the control and regulation of plant type PEPCs will be of great value to scientists developing strategies to cope with increasing uptake and accumulation of Cd in crop plants.

### 4.2 Did the lack of a PEPC isoenzyme affect plant health or plant response to Cd?

The purpose of the first portion of this study was to determine if the absence of one of three PEPC isoenzymes affected the seedlings' growth, response to CdCl<sub>2</sub> and health. To assess plant health I studied three different types of responses: physiological, photosynthetic, and relative Cd-concentration.

First, biomass and rosette diameter were measured to ascertain if the experimental CdCl<sub>2</sub> treatments inhibited plant growth. Overall, increasing concentrations of Cd inhibited biomass growth in all plant lines and the lack of any of the AtPPC isoenzymes resulted in a further decrease in size, even in control conditions. However lacking any of three AtPPC isoenzymes did not affect relative Cd-tolerance, the mutant lines were just as sensitive to Cd as the wild-type line. Treatment with Cd resulted in smaller rosette diameters in both the wild-type and mutant lines, and mutant lines were smaller than wild-type, but only at low Cd concentrations (0-1 µM). Taken together, these two measurements indicate that Cd negatively affected plant growth. While there are no published reports of the morphological responses of AtPPC1 – AtPPC3 to toxic metals, these results are corroborated by a number of studies reporting reduced biomass of wild-type *Arabidopsis* grown in the presence of Cd (Lee et al., 2003; Wójcik and Tukiendorf, 2011, Columbus unpublished). Johansson et al. (2008) measured organic acid production in Scots pine (*Pinus sylvestris*) and raised the possibility that when under Cd stress, plants allocate more resources towards root exudates, and less to plant growth, as a possible defense against Cd-induced damage.

Neither lacking an AtPPC isoenzyme nor CdCl<sub>2</sub> treatment affected the chlorophyll a:b or F<sub>v</sub>/F<sub>m</sub> ratios. Chlorophyll a:b ratios in all plant lines and Cd treatments were low. This could be due to the low light intensities used in the growth protocol. Under low light levels plants produce more chlorophyll b relative to chlorophyll a resulting in a lower chlorophyll a:b ratio (Aluru et al., 2001; Tanaka and Tanaka 2005; ). Similar chlorophyll a:b ratios have been reported in *Arabidopsis* exposed to 100 µmol m<sup>-2</sup> s<sup>-1</sup> (Aluru et al., 2001). Since the chlorophyll a:b and F<sub>v</sub>/F<sub>m</sub> ratios did not differ among plant lines nor among concentrations of CdCl<sub>2</sub>, it can be postulated that the Cd concentrations used and absence of one plant type PEPC isoenzyme did not affect the flow of electrons through PS II or cause damage to the photosynthetic apparatus. This could be due in part to low concentrations of Cd used in this study. Previous research on sunflower (*Helianthus annuus*; Pankovic et al., 2000) and *Arabidopsis* (Columbus unpublished data) using comparably low Cd concentrations (0-10 µM) also reported non-detectable effects on maximum PS II photochemical efficiency. In contrast, other studies have reported PS II

inhibition in *Arabidopsis* (Maksymiec et al., 2007) and pea (Chugh and Sawhney, 1999) seedlings grown at high Cd concentrations (1-10 mM). These changes in PS II activity are thought to be a result of closure of the PS II reaction centers (Baszynki et al., 1980). A more recent study using rice has shown that when grown under high Cd concentrations (75  $\mu\text{M}$ ) there was a reduced  $F_v/F_m$  ratio but the amounts of PS II reaction center and light harvesting complex proteins were unaffected (Pagliano et al., 2006). Pagliano et al. (2006) also reported that, at high concentrations of Cd, there were reduced amounts of electron transfer from water to dichlorophenolindophenol versus diphenylcarbazide (an electron donor) to dichlorophenolindophenol, suggesting that Cd affects the photosystem's water splitting ability. Another possibility was postulated by Weigel (1985): lower  $F_v/F_m$  ratios in plants treated with high concentrations of Cd could be a result of reduced Calvin cycle activity, not direct damage by Cd to PS II. Under the conditions in my experiment, it can be concluded that the smaller seedling mass and diameter were not due to Cd-induced photosynthetic damage.

All seedlings treated with  $\text{CdCl}_2$  took up Cd, with greater concentration in roots than in shoots, even when taking differences in biomass and rosette diameter into account. This pattern could be explained if *Arabidopsis* had mechanisms in their roots that minimized translocation of Cd to shoots or increased internal compartmentalization of Cd within root tissues. The increasing Cd concentration with increased  $\text{CdCl}_2$  in solution could explain the decrease in plant biomass and rosette diameter. The lack of an effect of Cd on  $F_v/F_m$  and chlorophyll a:b ratios may in part be due to intracellular defensive mechanisms, such as chelation of toxic metals. One of the best documented cases of metal chelation and detoxification is with Al toxicity. Buckwheat exudes oxaloacetic acid in response to Al stress, where it forms a non-toxic Al-oxaloacetate complex that is then transported and safely stored in leaves (Ma et al., 1997). As a result  $\text{Al}^{3+}$  is removed from the rhizosphere as well as plant roots leading to external and internal detoxification. So, understanding if and how organic acid production is regulated under Cd stress is essential to comprehending Cd tolerance in *Arabidopsis* and, by extension, other  $\text{C}_3$  plants.

### 4.3 How important is the PEPC pathway to reducing Cd stress?

The second half of this study investigated Cd-induced changes to the PEPC pathway. This was achieved by measuring transcript abundance for each of the AtPPC and AtPPCK isoenzymes, relative PEPC phosphorylation, relative PPCK activity, PEPC activity and abundance of specific organic acids.

In all plant lines there was an increase in relative transcript abundance in response to Cd for *AtPPC1* – *AtPPC3* as well as *AtPPCK1* – *AtPPCK2*, with relative transcript abundance in roots being greater than that found in shoots. The higher up-regulation in root tissue corresponds with the increased Cd concentration found in roots tissues. Previous research on P<sub>i</sub>-starved Arabidopsis noted up-regulation of only *AtPPC1* and *AtPPC2*, but not *AtPPC3*, as well as *AtPPCK1* – *AtPPCK2* (Gregory et al., 2009). Gregory et al. (2009) also reported greater transcript abundance in roots versus shoots in stressed plants. Studies of AtPPC isoenzymes under drought stress yielded a different expression pattern. Sánchez et al. (2006) discovered that while there was no significant up-regulation of *AtPPC1* – *AtPPC3*, there was a 4-fold up-regulation of relative *AtPPC4* transcript abundance in Arabidopsis roots experiencing a simulated drought stress. Differential induction of Arabidopsis PEPC genes has also been noted in salt stress studies. In three week old Arabidopsis subjected to 170 mM NaCl, root *AtPPC1* and *AtPPC3* were up-regulated after two to three days but up-regulation of root *AtPPC4* did not occur until after four days of salt stress (Sánchez et al., 2006). Both the P<sub>i</sub>-deficiency and salt studies measured *AtPPC* transcript abundance in roots only. While my study measured the effects of Cd after seven days of exposure, it is possible that, if measured at earlier time-points, there could have been a differential response.

When subjected to drought, mutants with reduced *AtPPC4* expression had up-regulation of the genes for the three other PEPC isoenzymes (Wang et al., 2012). This indicates that the absence of one isoenzyme may result in increased production of the remaining isoenzymes when the plant is under an abiotic stress. This phenomenon occurred in my study, but the specific isoenzyme with increased transcript abundance varied among the mutants. In the Cd-treated *Atppc1* plant line, there was less *AtPPC3* transcribed in roots

and more root and shoot *AtPPC2* relative transcript abundance when compared to the wild-type. Plants lacking *Atppc2* had greater up-regulation of *AtPPC1* transcripts in shoots but reduced abundance of *AtPPC3* transcripts in comparison to wild-type control. Plants lacking *Atppc3* had the lowest *AtPPC1* transcript abundance of the four lines but an increase in *AtPPC2* transcript abundance in both shoots and roots when treated with Cd. It is possible that lacking one of the PEPC isoenzymes adversely affects the plant's ability to function, forcing Arabidopsis to up-regulate the genes coding for the other PEPC isoenzymes in order to compensate. The reduced transcription of *AtPPC3* in both *Atppc1* and *Atppc2* mutants and the reduction of *AtPPC1* in *Atppc3* mutants, in comparison to wild-type *AtPPC* transcript abundance, indicates the possibility that *AtPPC1* and *AtPPC3* are both required for plants to fully function, especially when under Cd-stress.

Most of the research on AtPPCK isoenzyme transcript abundance has centered on non-metal studies. Gregory et al. (2009) reported an up-regulation of *AtPPCK1* and *AtPPCK2* transcripts in Pi-starved Arabidopsis versus non-starved seedlings, with greater transcript abundance in the roots than the shoots. These results mirrored responses measured in my study; however, differential responses were noted among the mutant lines. In comparison to wild-type, *AtPPCK1* was significantly down-regulated in the roots of all three mutant plants. It is possible that the lack of one of the PEPC isoenzymes is reducing the need for PEPC phosphorylation and thus PPCK activity. Research on *AtPPCK1* mutants reported those mutants with reduced (*din1*) or no (*csi8*) light-induced PEPC phosphorylation also had reduced rosette diameter (Meimoun et al., 2009). *Din1* and *sci8* mutants also had reduced concentrations of TCA cycle intermediates, such as succinic and fumaric acid, but no significant differences in glutamate, glutamine, or citric and malic acids (Meimoun et al., 2009). This suggests that inhibition of AtPPCK1 and subsequent phosphorylation of PEPC may not affect the portion of the TCA cycle dedicated to amino acid synthesis. Since glutamine and glutamate were unaffected, the PEPC pathway portion of the TCA cycle is maintained, whereas the reductions in succinic and fumaric acid indicated that the TCA cycle as a whole was not functioning correctly. Any changes noted in PEPC phosphorylation or PPCK/PEPC activity should help to explain how the TCA cycle is regulated by plants in response to CdCl<sub>2</sub> treatment.



Relative phosphorylation of p107 subunits of PEPC in roots of wild-type and mutant lines increased with increasing Cd concentrations. Interestingly, in shoots there was far less relative phosphorylation in the *Atppc2* and *Atppc3* mutants. In roots, relative phosphorylation of *Atppc3*'s PEPC was greater than that of the wild-type when treated with CdCl<sub>2</sub>. This indicates that the AtPPC3 isoenzyme, and to a lesser extent AtPPC2, may play a greater role in Arabidopsis' defense against CdCl<sub>2</sub>. Meanwhile, there was no difference in PEPC activity responses among the plant lines. So, while there was a decrease in relative phosphorylation of PEPC in the shoots of all mutants but an increase in roots of *Atppc2* and *Atppc3* mutants, there was no corresponding decrease in PEPC activity within each plant line. The general response of increased pSer-11 phosphorylation and subsequent increase in PEPC activity has been reported in Pi-starved root and shoot tissues of Arabidopsis (Gregory et al., 2009), developing castor oil plant endosperm (Gennidakis et al., 2007), P<sub>i</sub>-deficient white lupin proteoid roots (Uhde-Stone et al., 2003), as well as harsh hakea (*Hakea prostrata*) proteoid roots (Shane et al., 2013). The change in phosphorylation of some isoenzymes with no corresponding change in overall PEPC activity in the mutant lines I used could be explained by a differential response of the PEPC isoenzymes. The absence or decreased activity of one could result in increased activity of the remaining PEPC isoenzymes and, overall, there would not be a measurable difference total PEPC activity.

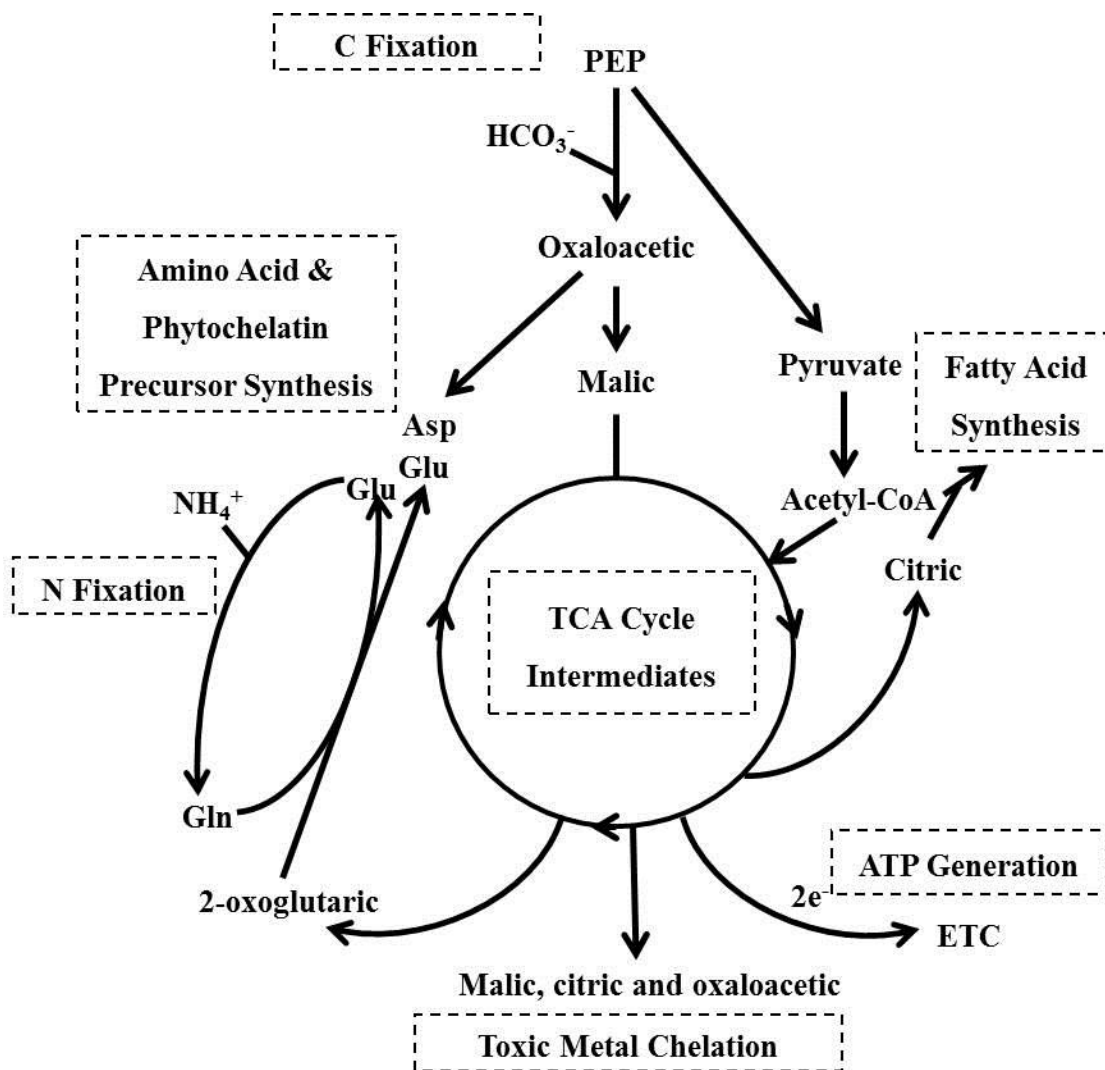
Sanchez et al. (2006) reported higher PEPC activity in the roots versus shoots of wild-type Arabidopsis grown under control conditions but they did not measure relative PEPC phosphorylation. In C<sub>4</sub> plants, PEPC phosphorylation is inherently linked to the light:dark cycle. When castor oil plant leaves were placed in darkness, PEPC phosphorylation decreased over time but could be reversed if the plant was illuminated (Murmu and Plaxton, 2007). Taybi et al. (2004) reported that PEPC was reversibly phosphorylated by PPCK in four different *Clusia* species. PEPC activity is linked to phosphorylation and subsequently PPCK in response to drought, salt stress, and Pi-starvation; the results of my Cd-PEPC study follow the same pattern.

Surprisingly, there was no difference in relative PPCK activity among plant lines or Cd treatments. This could be due to the short period of time in which the AtPPCK in the

samples could phosphorylate PEPC. Relative phosphorylation was allowed to accumulate over a period of seven days, while the AtPPCK assay lasted only 15 minutes. It could also be that the abundance of AtPPCK protein in the extracts was relatively low. Overall, AtPPCK relative activity was greater in roots than shoots, which could explain the corresponding Cd-induced increase in PEPC activity within Arabidopsis roots. In wild-type and the *Atppc1* line, relative phosphorylation was greater in shoots but AtPPCK activity was greater in roots. This difference could be explained by PEPC turnover in the roots, the first tissue to encounter Cd. Root PEPC has higher activity and could have greater turnover. While not measured in this study, protein turnover is one of the post-translation regulators of PEPC (Lepiniec et al., 1994). PEPC in wild-type and *Atppc1* shoots may have a slower turnover, and therefore relative phosphorylation is able to accumulate whereas PEPC in the roots undergoes a faster turnover, resulting in lower accumulated relative phosphorylation of PEPC. With *Atppc2* and *Atppc3* lines there was an opposite pattern: greater relative phosphorylation in roots, with *Atppc3* plants having greater relative rates than wild-type plants but no difference in relative shoot PEPC phosphorylation. It is possible that the AtPPC3 isoenzyme, and to a lesser extent the AtPPC2 isoenzyme, play an important role in leaf metabolism when plants are Cd-stressed.

Furthermore, PPCK is regulated by rapid turnover, not by secondary messengers such as  $\text{Ca}^{2+}$  or by phosphorylation (Jeanneau et al., 2002). It could be postulated that AtPPCK turnover rates increase in Cd-stressed Arabidopsis, which would explain the greater *AtPPCK1* and *AtPPCK2* transcript abundance, increased relative phosphorylation in roots, but no measurable difference AtPPCK relative activity because the amount of AtPPCK was quickly turned over.

Increased production of TCA cycle organic acids in response to initiation of the anapleurotic PEPC pathway can explain how plants respond to abiotic stress, such as Cd toxicity, which is summarized in Figure 4-1. A number of factors result in consumption of organic acids and the need for increased production:



**Figure 4-1 Fates of TCA cycle intermediates (organic acids) in Arabidopsis**

Organic acids play a role in C and N fixation, amino acid, phytochelatin precursor, and fatty acid synthesis as well as chelators used to either render metals unavailable for uptake or facilitate the uptake of essential and non-essential nutrients, as well as TCA cycle intermediates resulting the formation of NADH and electrons used in the electron transport chain (ETC) to generate ATP. Abbreviations include, glutamine (Gln), glutamate (Glu), aspartate (Asp). Drawn using information from Plaxton and Podestá (2006).

(1) Toxic metals, such as Cd, in the rhizosphere induce the production and exudation of metal-chelating organic acids such as malic, citric or oxaloacetic acid. Metal-chelate complexes in the rhizosphere can immobilize metals making them unavailable for uptake or facilitate metal uptake into the plant's roots and their subsequent storage in vacuoles.

(2) Precursors and intermediates of the TCA cycle are utilized by the plant to produce essential amino acids that could be used to repair damage caused by Cd stress. For example, transamination of pyruvate, oxaloacetate or 2-oxoglutarate can yield alanine, aspartate and glutamate, respectively (Berg et al., 2002). Aspartate can give rise to asparagine, methionine, threonine and lysine (Berg et al., 2002).

(3) Glutamate is a very important amino acid. It is involved in nitrogen (N) assimilation and acts as a precursor for other amino acids. Ammonia assimilation involving glutamine synthetase (GS; EC 6.3.1.2) and glutamine oxoglutarate aminotransferase (GOGAT; EC 1.4.1.13) results in the production of glutamate (Rhodes et al., 1980) Glutamate can give rise to asparagine, methionine, threonine and lysine (Berg et al., 2002). As previously mentioned, glutamate is one of the substrates involved in glutathione synthesis. Glutathione is a precursor to phytochelatins, which have been shown to have a high affinity for  $\text{Cd}^{2+}$  within plant tissues.

(4) Organic acids are also involved in fatty acid synthesis. Citric acid facilitates the transport of Acetyl-CoA, a precursor of the TCA cycle, across the mitochondrial membrane and into the cytoplasm, where Acetyl-CoA is used to form fatty acids. Once in the cytosol, citric acid reacts with ATP and coenzyme A to synthesize acetyl CoA, oxaloacetic acid and ADP. Organic acids are also involved in the storage of atmospheric carbon (C) in  $\text{C}_4$  and CAM plants (Buchanan et al., 2000). During periods of toxic metal stress, plants may increase C storage to replace the sucrose and starch used for cellular repair as well as increase the production of TCA cycle intermediates lost to amino acid synthesis, fatty acid synthesis, root exudation or degradation.

As TCA cycle intermediates, the primary role of organic acids is the production of NADH, which is then oxidized by the electron transport chain. This oxidation is linked to the chemiosmotic generation of ATP (Buchanan et al., 2000; Berg et al., 2002), which

may in part be used to repair damage or invest in defensive mechanisms, such as the production of chelators, against free  $\text{Cd}^{2+}$ .

Since organic acids are inherently linked to the defense and repair of damaged cells, their relative abundance in root and shoot tissue would be expected to increase or remain at control values when the plant is continually exposed to  $\text{Cd}^{2+}$ . As previously mentioned, a number of studies have reported increased organic acid concentrations after multiple days of exposure to  $\text{Cd}^{2+}$  (Guo et al., 2007; Johansson et al., 2008; Adeniji et al., 2010; Xie et al., 2013). Experiments in which the concentrations of organic acids remained at control (or steady state) cannot be interpreted as showing no up-regulation of the TCA cycle. If the rate of replenishment matches the rate of consumption, then no net change in concentration would be measured. One could use stable isotopes (especially  $^{13}\text{C}$ ) to measure the rates of TCA cycle activity, and the fates of newly synthesized organic acids, in response to stress.

Different organic acids also can also play multiple roles within a plant. Citric, malic and oxaloacetic acid can act as amino acid precursors, which could lead to the up-regulation of organic acid synthesis when the plant is attempting to repair damage. The primary role of succinic and fumaric acid is to act as intermediates in the TCA cycle. Any increases in succinic or fumaric acid concentrations could mean that the plant is attempting to up-regulate the TCA cycle and produce more ATP through the electron transport chain.

In this study, the concentrations of organic acids varied between tissue types and among plant lines. It should also be noted that in this experiment that total organic acids were measured and that there might have been differences in organic acid concentrations among compartments in the tissues. All four plant lines in this study contained less organic acids (when measurable) in roots than in shoots. When comparing the mutant lines to wild-type controls, all but the *Atppc2* line and to a greater extent the *Atppc3* line had a differential production organic acids. *Atppc2* plants had higher concentrations of malic acid in roots and oxaloacetic and citric acid in shoots; when grown with  $5\ \mu\text{M}$   $\text{CdCl}_2$  the oxaloacetic acid concentrations in shoots were greater than those in wild-type shoots. In the *Atppc3* mutants, malic acid concentrations in shoots and citric acid

concentrations in shoots and roots significantly increased with greater  $\text{CdCl}_2$ , and oxaloacetic acid concentrations in shoots were greater than those found in wild-type shoots. One might think that these higher concentrations of potential metal chelators would confer an advantage to the mutant plants; however, these two lines had the smallest biomass. Perhaps these increases in organic acids were involved in repair mechanisms (through the production of amino acid precursors), which diverted resources from growth. The lack of AtPPC1 did not have a similar consequence; hence I believe AtPPC2 and AtPPC3 are more important in terms of the plant's tolerance to Cd-stress.

#### 4.4 Conclusions and future work

The interactions among plants, metals, and the surrounding environment continue to be a relationship that has not been fully elucidated, be it a thorough understanding of the mechanisms of metal uptake, sequestration or compartmentalization. Researchers studying plant-metal interactions can oversimplify the relationship by measuring only one portion of the pathway of interest or looking only at the end products. In the current literature, most plant-metal chelation studies follow a similar pattern; the addition of a metal leads to increased activity of the enzyme of interest resulting in the increased production of a chelator (be it organic acids or phytochelatins). This model fails to take into account how isoenzymes can react differently in response to metal stress.

Isoenzymes can play a differential role within plant tissues and their overabundance or absence can affect other isoenzymes, the biochemical pathway, or the system as a whole. The study of isoenzymes has been applied to research on  $\text{P}_i$  starvation (Moraes and Plaxton, 2000; Veljanovski et al., 2006; Gregory et al., 2009), drought (Sánchez et al., 2006) and saline stress (Sánchez et al., 2006; Wang et al., 2012) as well as  $\text{C}_4$  and CAM metabolism (Taybi et al., 2004; Meimoun et al., 2009) but, until now, not Cd toxicity.

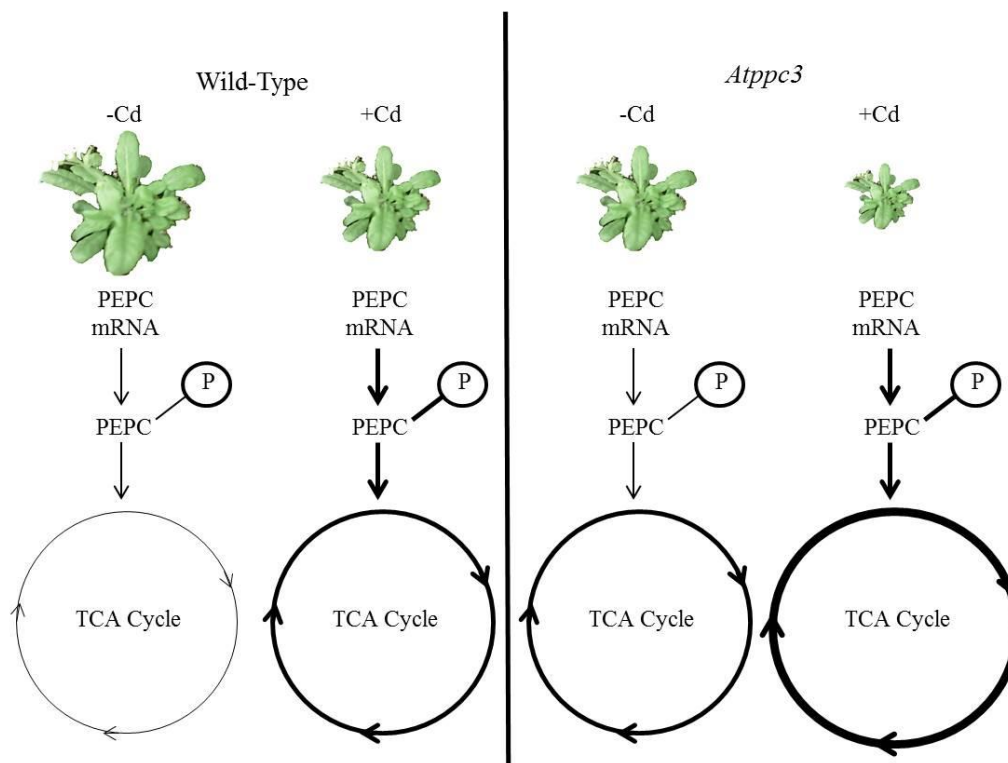
In this thesis, I studied PEPC isoenzymes under Cd stress with the use of Arabidopsis isoenzyme knockout mutants. In doing so, I have expanded upon the field of plant-metal interactions by discovering that; (1) each PEPC isoenzyme was expressed when Arabidopsis was exposed to Cd, (2) the absence of one isoenzyme can cause a differential response in the remaining isoenzymes, and (3) specifically, the absence of AtPPC3 and to

a lesser extent AtPPC2 has an adverse effect on Arabidopsis and its ability to cope with Cd stress.

The differential responses of mutants lacking AtPPC3 to Cd stress is summarized in Figure 4-2. First, in wild-type plants grown in the absence of Cd, relative PEPC isoenzyme transcripts, relative phosphorylation and PEPC activity remain low.

When grown in the presence of Cd, relative PEPC isoenzyme transcript abundance, phosphorylation and organic acid production increases. In contrast, plants lacking AtPPC3 are physically smaller and produce higher concentrations of organic acids compared to the wild-type plants. When Cd-stressed, mRNA transcript abundance, phosphorylation and activity increase but concentrations of some TCA cycle intermediates (oxaloacetic and citric) are greater than those found in Cd-treated wild-type plants. The smaller size coupled with increased abundance of organic acids in plants lacking AtPPC3 indicates that the lack of the AtPPC3 isoenzyme somehow inhibits the TCA cycle, leading to a build-up of intermediates or the increased production of amino acid precursors, even under control conditions. This situation is magnified when AtPPC3 mutants are Cd-stressed.

This research has given important insight into the Cd-isoenzyme response in Arabidopsis. I have clearly shown the value of integrating molecular techniques such as transcription studies with protein and enzymatic biochemistry and eco-physiological measurements to gain a better understanding of what is happening in the “big picture”, whereas a lack of integration of different techniques could result in the misinterpretation of how the organism is responding to a stressor.



**Figure 4-2 Model depicting response of wild-type versus *Atppc3* to Cd stress in Arabidopsis**

In wild-type Arabidopsis treated with  $\text{CdCl}_2$  there is an up-regulation in PEPC gene transcript abundance, relative phosphorylation of PEPC and increased production of organic acid TCA cycle intermediates. On the other hand plants lacking the AtPPC3 isoenzyme (*Atppc3*) were physically smaller than wild-type plants at similar Cd concentrations. While there was an up-regulation of mRNA transcripts, and relative phosphorylation (P) in Cd treated *Atppc3*, there was greater concentrations of organic acids (i.e. oxaloacetic) compared to wild-type plants in treatments lacking Cd and with Cd. Thicker lines represent increased amounts, concentrations and activities.



Future research on the investigations of phytochelatins and pathways controlling organic acid production could provide further clarification of how a plant regulates the synthesis of chelators. While not measured in this project, the TCA cycle intermediate 2-oxoglutaric acid is a precursor in glutamine synthesis, a known precursor of phytochelatins. It would be interesting to know if the PEPC pathway is the regulatory control for not only organic acids but also phytochelatin synthesis when plants are Cd-stressed. Studies of saline stress using lines without AtPPC4 have also raised the possibility that the presence or absence of the bacterial type PEPC could affect the regulation of the three plant type PEPC isoenzymes in Arabidopsis as well as responses to Cd. Knowledge of Cd-specific isoenzyme responses could be of interest to biotechnologists attempting to engineer transgenic plants for phytoremediation – plants exhibiting increased uptake of Cd could be chosen from phytoremediation. On the other hand, this knowledge could also be used by bioengineers in the creation of crop species that accumulate reduced concentrations of Cd in edible tissues.

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## Appendices

### Appendix A Composition of MS nutrient medium (Murashige and Skoog, 1962)

Component	Concentration (mg/L)
Ammonium nitrate	1650
Boric acid	6.2
Calcium chloride, anhydrous	332.2
Cobalt chloride•6H <sub>2</sub> O	0.025
Cupric sulfate•5H <sub>2</sub> O	0.025
Na <sub>2</sub> EDTA•2H <sub>2</sub> O	37.26
Ferrous sulfate•7H <sub>2</sub> O	27.8
Magnesium sulfate, anhydrous	180.7
Manganese sulfate•H <sub>2</sub> O	16.9
Molybdc acid (sodium salt) •2H <sub>2</sub> O	0.25
Potassium iodide	0.83
Potassium nitrate	1900
Potassium phosphate, monobasic	170
Zinc sulfate•7H <sub>2</sub> O	8.6
Glycine (free base)	2
Myo-Inositol	100
Nicotinic acid (free acid)	0.5
Pyridoxine•HCl	0.5
Thiamine•HCl	0.1

**Appendix B Arabidopsis PCR primers.**

Name	Primer direction	Sequence
<i>AtPPC1</i> <sup>1</sup>	Forward	'5-GCTGCTGGTCTACAAAACAC-3'
	Reverse	'5-AGCATGTGTTCAATGATCTCG-3'
<i>AtPPC2</i> <sup>1</sup>	Forward	'5-AAACGTTCTTGTACCATTCC-3'
	Reverse	'5-CATTA AAAAGCGTACGGGA-3'
<i>AtPPC3</i> <sup>1</sup>	Forward	'5-GTGAAAGAAAACAAAACCTTCG-3'
	Reverse	'5-CTTCCAGAAACAGAGTCAGTG-3'
<i>AtPPCK1</i> <sup>2</sup>	Forward	'5-GTTTTACGGCGAGACAG-3'
	Reverse	'5-ACA ACTCTGCTTTCTCACATC-3'
<i>AtPPCK2</i> <sup>2</sup>	Forward	'5-GGTTATCAGAATCAGAATCC-3'
	Reverse	'5-TGCTGAACACACA ACTACG-3'
<i>UBQ10</i> <sup>3</sup>	Forward	'5-CAGAACTTTGGCCGACTAC-3'
	Reverse	'5- ATGGTCTTTCCGGTGAGAG-3'

<sup>1</sup> (Sanchez and Cejudo, 2003)<sup>2</sup> (Fontaine et al., 2002)<sup>3</sup> (Divi et al., 2010)

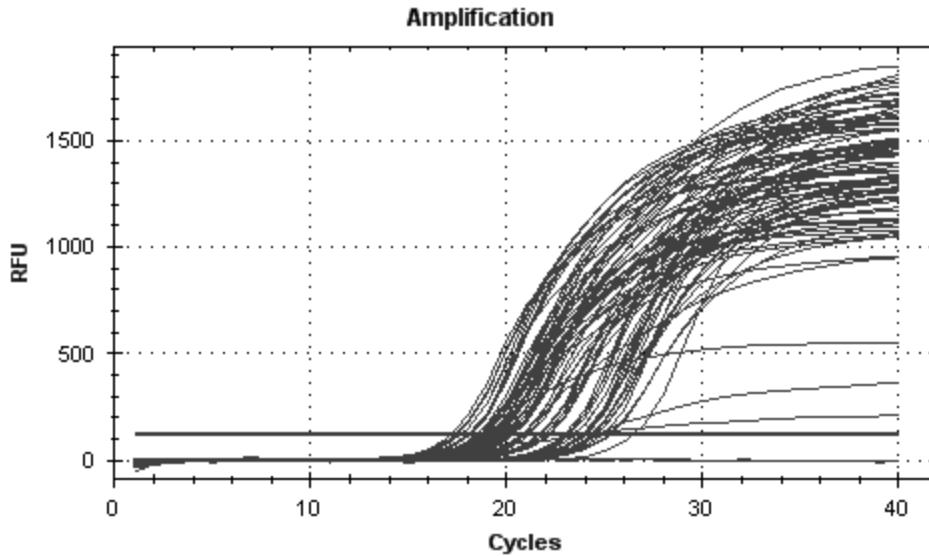


**Appendix C** Bradford Assay: total protein concentrations from Arabidopsis tissue used for equal protein loading

Plant Line	CdCl <sub>2</sub> [μM]	Tissue Type	PEPC Experiment Replicate			PCK Experiment Replicate		
			Concentration (μg/μl)			Concentration (μg/μl)		
			A	B	C	A	B	C
Wild-Type	0	shoot	1.67	1.43	1.27	0.95	0.90	0.98
Wild-Type	1	shoot	1.07	1.12	1.26	0.95	1.24	0.95
Wild-Type	5	shoot	1.28	1.26	1.21	0.91	1.08	0.82
<i>Atppc1</i>	0	shoot	1.28	1.23	1.17	0.89	0.94	0.90
<i>Atppc1</i>	1	shoot	1.23	1.12	1.19	1.02	1.02	0.95
<i>Atppc1</i>	5	shoot	1.27	1.30	1.20	1.36	1.17	1.14
<i>Atppc2</i>	0	shoot	1.48	1.16	1.33	1.15	1.14	1.12
<i>Atppc2</i>	1	shoot	1.49	1.34	1.38	1.13	0.66	1.02
<i>Atppc2</i>	5	shoot	1.19	1.40	1.39	1.09	1.09	1.35
<i>Atppc3</i>	0	shoot	1.26	1.35	1.30	1.21	1.36	1.30
<i>Atppc3</i>	1	shoot	1.28	1.16	1.01	1.33	1.42	1.35
<i>Atppc3</i>	5	shoot	1.18	1.46	1.22	1.40	1.11	1.08
Wild-Type	0	root	1.41	1.31	1.15	0.98	1.04	0.82
Wild-Type	1	root	0.92	1.02	0.94	0.87	1.24	0.81
Wild-Type	5	root	1.07	1.10	1.28	0.68	1.25	0.76
<i>Atppc1</i>	0	root	1.10	1.23	1.06	1.03	0.85	0.83
<i>Atppc1</i>	1	root	0.92	1.02	0.84	0.89	0.79	0.98
<i>Atppc1</i>	5	root	0.99	0.99	0.94	0.92	0.99	0.98
<i>Atppc2</i>	0	root	1.16	0.94	0.98	0.91	0.73	0.88
<i>Atppc2</i>	1	root	1.01	1.03	1.08	1.07	1.08	0.81
<i>Atppc2</i>	5	root	1.01	0.99	1.09	0.96	1.03	1.13
<i>Atppc3</i>	0	root	0.92	1.07	1.09	1.15	1.09	1.17
<i>Atppc3</i>	1	root	1.11	0.79	1.13	1.13	1.04	1.11
<i>Atppc3</i>	5	root	0.83	1.05	0.86	1.18	1.06	0.97

**Appendix D** Percentage recovery of organic acids from digested standards

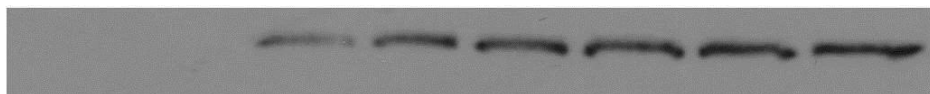
<b>Organic Acid</b>	<b>Recovered (%)</b>
Oxaloacetic	95.0 ± 2.4
Malic	89.7 ± 2.8
Citric	94.2 ± 2.9
Fumaric	88.2 ± 1.8
Succinic	88.7 ± 3.0



**Appendix E Example of RT-qPCR amplification curve from *AtPPC1* and *UBQ10* in Arabidopsis shoots**

Example of RT-qPCR amplification curve depicting transcript abundance of *AtPPC1* and *UBQ10* in Arabidopsis shoots after being exposed to one of the experimental Cd treatments over 40 cycles. Relative fluorescence units (RFU) are measuring SYBR green fluorescence in each reaction tube. Horizontal line at 125 RFU is the fluorescence threshold.

<b>MC-LR</b>	-	+	+	+	+	+	+	+
<b>Time (min)</b>	30	0	5	10	15	20	25	30



#### **Appendix F APS IgG trial to determine approximate assay time**

Immunoblot of APS IgG at varying times after the addition of MC-LR. 15 min was determined to be the best time for MC-LR incubation.

## Curriculum Vitae

**Name:** Ian Robert Willick

**Post-secondary Education and Degrees:** The University of Western Ontario  
London, Ontario, Canada  
2007-2011 B.Sc. (honours biology)

The University of Western Ontario  
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**Honours and Awards:** Royal Canadian Legion Award  
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2007

Scholarship of Distinction  
The University of Western Ontario  
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Graduate Research Scholarship  
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George H. Duff Travel Award  
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**Presentations:**

4. **Willick IR** and Macfie, SM (2012) Cadmium-induced stress in *Arabidopsis thaliana* triggers up-regulation of phosphoenolpyruvate carboxylase (PEPC) isoenzymes and expression of PEPC specific kinase (PPCK). Eastern Regional Meeting of Canadian Society of Plant Biologists. (**Poster presentation by IR Willick**). Waterloo, Ontario.
3. **Willick IR** and Macfie, SM (2012) Impact of cadmium-induced stress on phosphoenolpyruvate carboxylase isoenzyme concentrations in *Arabidopsis thaliana*. Annual Meeting of Canadian Society of Plant Biologists. (**Poster presentation by IR Willick**). Edmonton, Alberta.
2. **Willick IR** and Macfie, SM (2011) Response of phosphoenolpyruvate carboxylase to cadmium stress in *Arabidopsis thaliana*. Undergraduate Biology Research Day. (**Oral presentation by IR Willick**). London, Ontario.
1. **Willick IR** and Macfie, SM (2011) Response of phosphoenolpyruvate carboxylase to cadmium stress in *Arabidopsis thaliana*. Ontario Biology Day. (**Poster presentation by IR Willick**). Waterloo, Ontario.

<b>Academic Service</b>	Earth Day Colloquium Volunteer 2012/04 & 2013/04
	Biology Undergraduate Education Committee Graduate Student Representative 2012/08-2013/08
	Biology Website Committee Graduate Student Representative 2013/01-2013/08
<b>Professional Affiliations</b>	Canadian Society of Plant Biologists 2011-present