UNIVERSITY OF CALIFORNIA, SAN DIEGO

Long Distance Transport of Phytochelatins in Arabidopsis and the Isolation and Characterization of Cadmium Tolerant Mutants in Arabidopsis

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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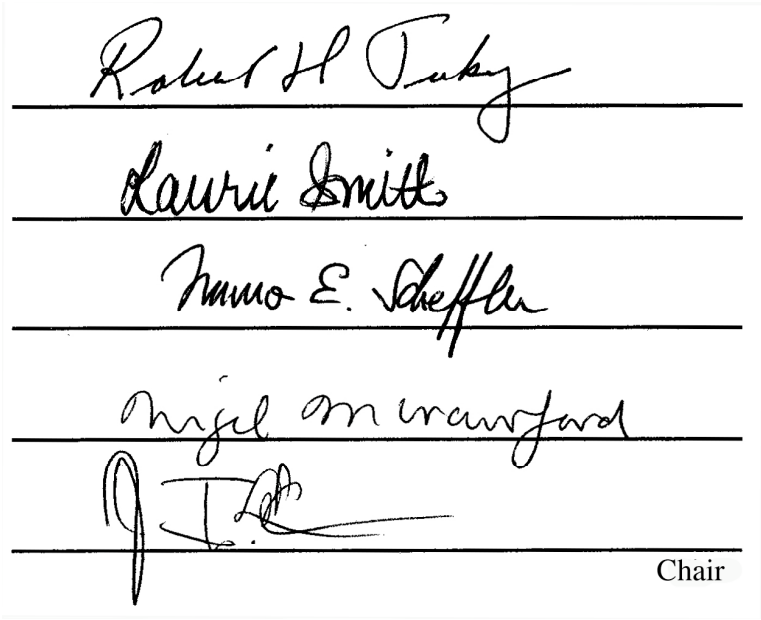
2005

The dissertation of Alice Chen is approved, and it is acceptable in quality and form for publication on microfilm:



Chair

University of California, San Diego

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The text of Chapter Three, in part, is a reprint of the materials that has been submitted for publication. The dissertation author was the primary researcher and

author and co-authors, Julian I. Schroeder and Elizabeth A. Komives, listed in this publication directed and supervised the research which forms the basis of this chapter

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Li, Y., Parkash, O., Carreira, L., Lee, D., Chen, A., Schroeder, J. I., Balish, R., Meagher., R.B., (2004) Overexpression of phytochelatin synthase leads to enhanced arsenic tolerance and cadmium hypersensitivity in *Arabidopsis*. *Plant Cell Physiology* 45: 1787-1797.

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ABSTRACT OF THE DISSERTATION

Long Distance Transport of Phytochelatins in Arabidopsis and
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by

Alice Chen

Doctor of Philosophy in Biology

University of California, San Diego, 2005

Professor Julian I. Schroeder, Chair

Heavy metal pollution is a global environmental concern. Plants have been discovered at contaminated sites, however, the molecular mechanisms that these plants employ to deal with heavy metal stress is not well characterized.

To gain a further insight into the genes and molecular pathways plants utilize to deal with cadmium stress, a post-germination screen was developed in the plant model system *Arabidopsis* to isolate mutants tolerant to high levels of cadmium. Several cadmium tolerant mutants were isolated from screening EMS mutagenized and activation tagged populations. *Cd11*, an EMS mutagenized mutant, displayed a significantly increased tolerance to cadmium in both shoot and root tissues in comparison to wild type and over-accumulated cadmium in both shoot and root tissues

in comparison to wild type. Several activation tagged mutants were also shown to exhibit increased tolerance to cadmium in both shoot and root tissues.

One of the primary mechanisms that plants employ for dealing with heavy metal stress is the production of phytochelatins. Phytochelatins are produced post-translationally by the enzyme phytochelatin synthase (PCS). *Arabidopsis* has two functional *AtPCS* genes. We have isolated a loss-of-function allele in the *AtPCS2* gene, *atpcs2-1*, and generated a double loss-of-function *AtPCS* mutant, *cad1-3 atpcs2-1*. *atpcs2-1* did not show an altered response to cadmium in comparison to wild type in either shoot or root tissues, cadmium accumulation, or levels of phytochelatins. *cad1-3 atpcs2-1* shared a similar response to cadmium as the single mutant *cad1-3* in shoot and root growth, cadmium accumulation, and the lack of detectable phytochelatins.

In addition, we have determined that phytochelatins were able to undergo long distance transport in a shoot to root direction. Fluorescence HPLC coupled to mass spectrometry analyses and detected the presence of phytochelatins in root tissues of transgenic plants expressing the wheat PCS (*TaPCS1*) gene under the control of a shoot specific promoter (*CAB2*) in the PC-deficient mutant *cad1-3*. Analyses demonstrated that CAB2::*TaPCS1*/*cad1-3* lines complement the cadmium and arsenic metal sensitivity of *cad1-3* shoots and over-accumulated cadmium in root tissues. Grafting experiments between wild type shoots and *cad1-3 atpcs2-1* roots also showed shoot to root PC transport.

I.

Isolation and Characterization of Cadmium Tolerant *Arabidopsis* Mutants

1. Abstract

As of to date, there have been few genes identified in plants that specifically confer cadmium tolerance. In order to isolate and identify genes involved in cadmium tolerance and detoxification, a post-germination screen was developed and performed in the plant model organism *Arabidopsis thaliana*. The populations mutagenized with the chemical mutagen ethyl methane sulfonate (EMS) or by random T-DNA insertions, also known as activation tagging were, screened to isolate cadmium tolerant mutants. Potential cadmium tolerant mutants were selected based on the ability to grow and maintain green cotyledons in comparison to wild type on plant media supplemented with 1.5 mM $CdCl_2$. Nineteen putative mutant lines were isolated from the populations mutagenized with EMS and rescreened for phenotype confirmation. *Cd11* displayed the highest percentage of green cotyledons in the presence of 1.5 mM $CdCl_2$ in comparison to the other EMS generated cadmium tolerant putative mutants. *Cd11* was selected for further analysis and showed over-accumulation of cadmium in both shoot and root tissues in comparison to wild type. *Cd11* also displayed enhanced shoot and root growth in the presence of cadmium in comparison to wild type. From the activation tagged lines, seventeen putative cadmium tolerant mutants were isolated and rescreened for phenotype confirmation. Four lines from the activation tagged populations were selected for further analysis and initial mapping experiments.

2. Introduction

Heavy metals are often classified as metals having a density equal or greater than 5.0 g/cm^3. Some heavy metals serve as essential nutrients at low concentrations, such as iron and copper, but all heavy metals become toxic at high concentrations. As plants are sessile organisms and cannot remove themselves from contaminated environments, plants have developed strategies for coping with heavy metal toxicity. However, the molecular mechanisms that plants employ to detoxify heavy metals from their environment remains relatively unknown.

Heavy metal pollution is a global environmental concern as metals cannot be biologically or chemically degraded, and hence are indestructible. Traditional means of remediation, which involves the physical removal of the heavy metal contaminated soil, is both labor intensive and costly. Phytoremediation, which is the usage of plants to remove pollutants from the environment, is an attractive alternative to these traditional remediation methods. Several plants have been discovered at heavy metal contaminated sites that posses the unique ability to survive toxic conditions. In addition, some of these plants have been found to accumulate a significant percentage of their biomass in heavy metals and are called hyperaccumulators. Examples of natural hyperaccumulators include the arsenic hyperaccumlating fern species *Pteris vittata* (Gumaelius et al., 2004) and the nickel hyperaccumulating species *Thlaspi caerulescens* (Freeman et al., 2004). However, many of these natural hyperaccumulators are not ideal for phytoremediation due to their small size, slow

growth, slow accumulation of heavy metals, and inability to survive different ecotypes. Therefore an understanding of the molecular mechanisms higher plants employ to remove, accumulate, and detoxify heavy metals is required for phytoremediation to be successful. In addition, an understanding of the relationship between compounds found in soil and plant heavy metal uptake is also a requirement for phytoremediation. The degree to which higher plants are able to take up heavy metals depends on the concentration of heavy metals in the soil, bioavailability of the metals, which is modulated by the presence of organic matter, pH, redox potential, temperature, and concentrations of other elements (Das et al., 1998).

Cadmium is a significant heavy metal pollutant, due to its greater solubility and high toxicity. Cadmium (Cd) is a relatively rare transition metal with a density of 8.6 g/cm^3. Cadmium's most common oxidation state is +2, although rare examples of +1 have been found. The main sources of cadmium in the air are the burning of fossil fuels such as coal or oil and the incineration of municipal waste (http://www.epa.gov/ttn/atw/hlthef/cadmium.html). Cadmium is often released into the environment from urban traffic, cement factories, and as a by-product of phosphate fertilizers (Toppi and Gabbrielli, 1999). In areas with low anthropogenic pressure, cadmium can be released as a result of rock mineralization processes (Baker et al., 1990). In non-polluted soils it is estimated that the concentrations of cadmium range from 0.04 to 0.32 μM. Soils containing concentrations from 0.32 to 1 μM can be classified as moderately polluted (Wagner, 1993).

Cadmium is one of the few elements that have no discerniable purpose in human health and is in fact extremely toxic even at low concentrations. Cadmium often interferes with zinc, magnesium, and calcium containing enzymes by displacing these elements and acting as a very poor substitute for these elements. Short-termed exposure to of cadmium in humans through inhalation mainly effects the lungs, resulting in pulmonary irritation and increased lung cancer risks. Long-term exposure through inhalation or oral exposure to cadmium leads to a build-up of cadmium in the kidneys that can cause kidney and liver disease (Friberg et al., 1986). Cadmium poisoning is the cause of the itai-itai disease in which patients suffer from severe osteoporosis and oasteomalacia, in addition to kidney damage (Inaba et al., 2005). Cadmium has also been found to be teratogenic, carcinogenic, and mutagenic, for a large number of animal species (Degraeve, 1981).

In plants, cadmium competes with the physiological transport of nutrients such as calcium, iron, magnesium, manganese, copper and zinc. As cadmium is a nonessential element and therefore is transported by transmembrane nutrient transporters (Clarkson and Lüttge 1989; Riveta et al., 1997; Clemens et al., 1998; Grotz et al., 1998; Curie et al., 2000; Picard et al., 2000; Thomine et al., 2000; Clemens et al., 2002; Thomine et al., 2003; Papoyan and Kochian 2004). Cadmium is believed to penetrate the root through the cortical tissue, reach the xylem through an apoplastic and/or symplastic pathway, and may be complexed by several ligands such as organic acids and/or phytochelatins (Salt et al., 1995). For most plants, cadmium tends to accumulate in the roots with very little being transported into the shoots

(Cataldo, 1983). The roots are the first tissue to experience cadmium toxicity, and cadmium has been found to damage nucleoli, alter the synthesis of RNA, inhibit ribonuclease activity (Shah and Dubey, 1995), reduce absorption of nitrate by inhibiting the nitrate reductase activity in shoots (Hernandez, 1996). In shoot tissues, cadmium leads to Fe (II) deficiency, which affects photosynthesis (Alcantara et al., 1994). Shoot tissues become chlorotic and exhibit reduced growth due to inhibition of the elongation growth-rate processes from an irreversible inhibition on a proton pump (Aidid and Okamoto, 1993). Cadmium damages the photosynthetic apparatus, in particular the light harvesting complex II (Krupa, 1988) and photosystems I and II (Siedlecka and Krupa, 1996). Cadmium lowers the total chlorophyll content, cartenoid content, and increases non-photochemical quenching (Larsson et al., 1998). Chlorosis from excess cadmium appears to be either a direct or indirect interaction of cadmium with foliar iron (Larsson et al., 1998).

To date, there have been no identified plant genes that specifically confer cadmium tolerance. A post-germination screen was developed in *Arabidopsis* to allow for the rapid screening of a large, mutagenized population of *Arabidopsis* seeds to isolate mutants with a high tolerance to cadmium. The post-germination screen utilizes a fine nylon mesh to aid in the rapid transfer of a large number of seedlings. Seedlings were first germinated on a porous nylon mesh and agar plates containing only plant nutrients. After four days of growth, the porous nylon mesh and seedlings were then transferred to plates containing high concentrations of cadmium. As cadmium causes chlorosis in plants, seedlings that were able to maintain green

cotyledons after four days on 1.5 mM $CdCl_2$ were selected as a putative cadmium tolerant mutant. The relatively simple visual scoring for green cotyledons allowed for the rapid screening of a large population of mutagenized seeds.

3. Methods

CADMIUM POST-GERMINATION TOLERANCE SCREEN

M2 Ethyl Methane-Sulfonate (EMS) mutantgenized populations of Columbia ecotype *Arabidopsis thaliana* seeds obtained from Lehle Seeds (Lehle Seeds, TX) and T2 of Columbia ecotype *Arabidopsis thaliana* transformed with the activation tagging vector pSKI015 (GenBank Accession #AF187951), which were generously provided by Wolf Scheible (Stanford University) and Detlef Weigel (Max Planck Institute, Tübingen), were used for screening. Seeds from the EMS and activation tagged populations were sterilized, placed on square petri dishes containing minimal media (quarter strength MS, 1 mM MES, 1% Agar Type A), with a sheet of Spectrum Nylon mesh containing 200 μM pores separating the seeds from the agar. The minimal media was designed to allow plant growth, but also to reduce the content of nutrients that can form complexes with cadmium and/or compete in their uptake. After acclimation at 4ºC for two days, the plates were transferred to a growth chamber (24 hour/day light exposure) at 23ºC, where the seedlings were grown horizontally for four days. After four days post germination, in which the cotyledons have emerged, the nylon mesh and seedlings were transferred under sterile hood conditions to a MS agar plate containing the minimal media supplemented with 1.5 mM $CdCl_2$ for an

additional four days. Mutants displaying green cotyledons after 4 days of 1.5 mM cadmium treatment are then washed with 100 mM $CaCl_2$ for five minutes in order to displace the cadmium from cell walls of the seedlings. Putative mutants were then plated on cadmium free minimal media for recovery for two days before transfer to soil. Seeds were collected, sterilized, and used for rescreening under the same conditions as the original mutant screen (4 days on 0 μM $CdCl_2$ agar plates and an additional 4 days on 1.5 mM $CdCl_2$ agar plates).

PLANT MATERIAL, GROWTH CONDITIONS, AND METAL STRESS TREATMENTS

For growth in Petri dishes, *Arabidopsis* seedlings were grown on one-quarter-strength Murashige and Skoog (MS) basal medium (Sigma), 1 mM MES, 1% agar and the indicated concentrations of heavy metals. Seedlings used for shoot growth analyses and those used for root analyses were grown vertically under 24 hour light condition.

Plants used for Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) were grown in 80 mL of hydroponic medium as described (Arteca and Arteca 2000) with minor modifications: Plants were germinated in Petri dishes and grown vertically for the first four days and subsequently moved to a horizontal position for an additional two days to bend the hypocotyl to aid in successful transplantation onto the hydroponic sponge. Plants were grown on sponges (Jaece Industries, Inc., North Tonawanda, New York; catalog #L800-B; www.jaese.com) in

Magenta boxes (Sigma, Catalog #V 8505) at 24°C under a 16/8-h day/night period and hydroponic media were replaced every 3-4 days. After reaching the bolting stage (approximately 4 week old plants), the hydroponic medium was replaced with 50 mL of the same media to which 20 μM $CdCl_2$ were added for a period of three days for cadmium accumulation analyses.

For analysis of Cd^{2+}-dependent root growth, seeds were sterilized and plated on plates containing 25% MS medium, 1 mM MES, 1% Agar Type A (Sigma), cold treated at 4 ºC for 48 hours, and grown vertically under 24 hour light growth room conditions for five days. Seedlings were then transferred to 25% MS, 1 mM MES, 1% Agar plates containing 20 μM $CdCl_2$ for an additional 72 hours of vertical growth.

ICP-OES

To measure metal accumulation, plants are grown under hydroponic conditions in hydroponic media (12.5 mM KNO_3, 6.25 mM KH_2PO_4, 5 mM $MgSO_4$, 5 mM $Ca(NO_3)_2$, 125 μM Fe-EDTA, 3.5 mM H_3BO_3, 1.1 mM $MnCl_2$, 100 μM $ZnSO_4$, 12.5 μM $NaMoO_4$, 500 μM NaCl, 900 μM $CoCl_2$) until bolting stage. Cadmium treated plants grown under hydroponic conditions were first washed and then separated into root and shoot tissues. Shoot tissues were separated immediately below the cotyledons and above the hydroponic sponge. Root tissues were separated below the hydroponic sponge, approximately 3 cm below the cotyledons. Shoots were rinsed three times in deionized water. For ICP-OES analyses of root tissue, roots were rinsed in deionized water, washed in 100 mL of 100 mM $CaCl_2$ on an orbital shaker (Bellco

Glass, Inc., NJ) for 5 minutes at approximately 135 rpm (speed setting #4), and then washed in 100 mL of deionized water on the orbital shaker (speed setting #4) for an additional three minutes. Both shoot and root tissues were dried at 60ºC overnight. The dry weight was recorded, and then the tissues were digested in 70% trace metal grade nitric acid (Fisher Scientific) overnight. Samples were then boiled for 30 minutes to ensure complete digestion and diluted to a final concentration of 5% nitric acid with deionized water.

SOUTHERN BLOT ANALYSIS

Genomic DNA was extracted from leaves of *Cd29, Cd37, Cd47, Cd52, Cd54*. Approximately 2.5 μg of genomic DNA was digested overnight with either the restriction enzyme EcoRI or HindIII. The digested DNA was concentrated and separated on a 0.7% agarose gel containing 0.5% ethidium bromide, transferred to a nylon membrane, and UV light cross-linked. The blot was probed with pBlueScript vector, which was used to construct the activation tagging vector pSKI015, and detected using Kodak film with an overnight exposure at –80ºC.

TAIL-PCR

DNA fragments flanking the activation tag were obtained by a series of three nested TAIL-PCR reactions (Liu et al., 1995). The PCR reactions were performed with the degenerate primer AD2- NGTCGA(G/C)(A/T)GANA(A/T)GAA in combination with primers specific for the pSKI015 activation tagging vector (TR1

A A C C T T G A C - A G T G A C G A C A A A T C G , TR2 CGAATTTTGCGACAACATGTCGAG, and TR3 ATCGTGAAGTTTCTCATCTAA GCC). The initial reaction, to amplify the genomic DNA with the TR1 primer and one of the degenerate AD primers, was run under the following conditions (94°C 1 min, 5 x (94°C 30 sec, 62°C 1 min, 72°C 2.5 min), 2 x (94°C 30 sec, 25°C 3 min, ramping at 33% speed to 72°C 2.5 min), 15 x (94°C 30 sec, 68°C 1 min, 72°C 2.5 min, 94°C 30 sec, 44°C 1 min, 72°C 2.5 min), 72°C 5 min). The second reaction, to amplify the product of the first PCR reaction, utilized the TR2 primer with the degenerate primer, and was run under the following conditions: (15 x (94°C 30 sec, 64°C 1 min, 72°C 2 min, 94°C 30 sec, 44°C 1 min, 72°C 2 min), 72°C 5 min). The last nested PCR reaction was run using the product of the second reaction, and used the TR3 primer with the degenerate primer. Conditions for the final PCR reaction were as follows: (20 x (94°C 30 sec, 44°C 1 min, 72°C 2.5 min), 72°C 5 min). The products of the final PCR reaction were subcloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced (Retrogen, San Diego). The sequenced product was then examined using BLAST analysis (Altschul et al., 1997) against the *Arabidopsis* sequence database to determine the location of the activation tag.

4. Results

DEVELOPMENT OF A POST-GERMINATION SCREEN FOR CADMIUM TOLERANT MUTANTS

To date no plant genes that specifically confer cadmium tolerant has been reported, therefore a cadmium tolerant mutant was developed to isolate putative cadmium tolerant mutants in the plant model system *Arabidopsis thaliania*. High concentrations of cadmium inhibits seed germination (data not shown). The screen was initially developed to screen for individuals that displayed the ability to germinate on 1 mM $CdCl_2$. Under these conditions, eight putative cadmium tolerant mutants were isolated from populations mutagenized with EMS. However none of the individuals isolated from the germination screen survived to produce seeds. Therefore the germination screen was unsuccessful in isolating cadmium tolerant mutants.

The screen was then modified to a post-germination screen. Figure I-1 is an illustration of the post-germination screen in which seeds were germinated on a nylon mesh with a large pore size of 200 μm and minimal media plates for a period of four days (Figure I-1A) and then transferred with the aid of the nylon mesh to minimal media plates containing 1.5 mM $CdCl_2$ (Figure I-1B). 1.5 mM $CdCl_2$ was determined to be the cadmium concentration that inhibited the ability of a large percentage of wild type seedlings to maintain green cotyledons (Figure I-2). Approximately 7.5% of wild type seedlings were able to maintain green cotyledons after four days on 1.5 mM $CdCl_2$. Plates used for the post-germination screen contained minimal media to increase the bioavailability of cadmium. As cadmium normally inhibits growth and photosynthesis in plants (Aidid and Okamoto, 1993), seedlings were assayed for their ability to retain green cotyledons and undergo further development after a period of four days on 1.5 mM $CdCl_2$. The modification of the screen to a post-germination

screen allowed for the rapid isolation of several putative mutants that were able to survive and produce seeds.

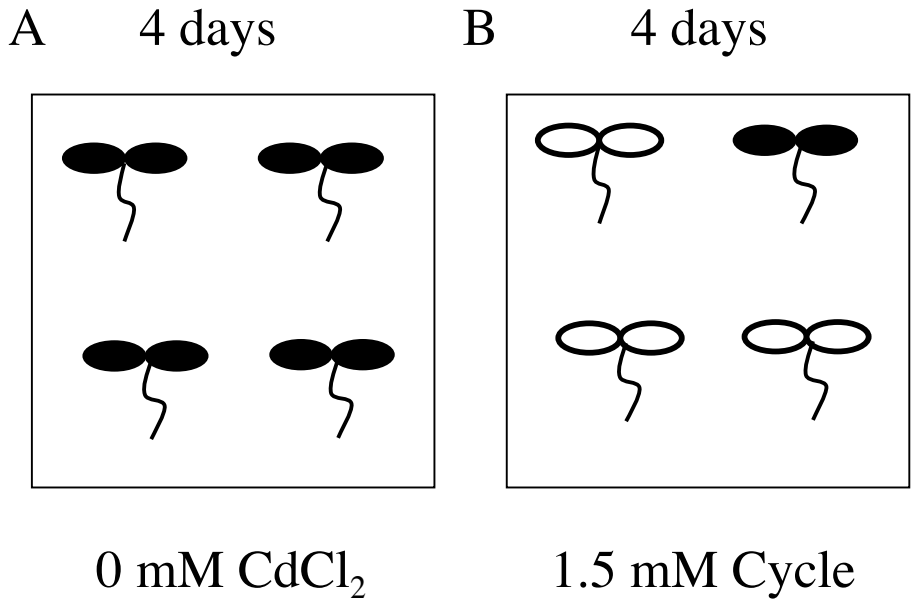


Figure I-1: Diagram of post-germination screen for isolation of cadmium tolerant mutants.
(A) Left panel shows germination and growth on 0 μM $CdCl_2$ minimal media plates with nylon mesh containing a pore size of 200 μm. Cotyledons of the seedlings are shown in black to indicate chlorophyll production.
(B) Right panel shows seedlings transferred with the aid of the porous nylon mesh from the 0 μM $CdCl_2$ plates onto minimal media plates supplemented with 1.5 mM $CdCl_2$. Upper right seedling with black cotyledons is represented as a putative cadmium tolerant mutant that has retained its chlorophyll content. The upper left, lower left, and lower right seedlings with the white cotyledons represent chlorosis.

GROWTH RESPONSE OF *CD11* IN THE PRESENCE OF CADMIUM

After screening approximately 8,400 EMS mutagenized lines, nineteen putative mutants were isolated and subjected to underwent rescreening for phenotype confirmation (Figure I-2). Rescreening conditions were the same conditions used for the original post-germination screen (See Materials and Methods). *Cd11* had the highest percentage of green cotyledons (76%; Figure I-2) in comparison to the other eighteen putative mutants and was selected for further characterization.

Cd11 shoot growth tolerance to cadmium was further characterized repeating the cotyledon assay on a wider range of cadmium concentrations. *Cd11* was exposed to 0, 0.5, 1.5 and 3.0 mM of $CdCl_2$ (Figure I-3). Both wild type and *Cd11* were able to germinate and grow normally in control plates not supplemented with cadmium (Figure I-3). On all tested concentrations of cadmium (0.5, 1.5, and 3.0 mM $CdCl_2$), *Cd11* displayed a higher percentage of green cotyledons in comparison to wild type (Figure I-3). Therefore *Cd11* displays greater cadmium tolerance in shoot tissues in comparison to wild type.

Due to genetic variability between individual seedlings, a small percentage of wild type individuals had the ability to maintain green cotyledons (Figure I-4B). In addition, a small percentage of individuals in the *Cd11* population were sensitive to cadmium and displayed chlorosis in their cotyledons (Figure I-4A). Therefore in each population there appeared to be a small percentage of individuals with an altered response to cadmium due to natural genetic variability.

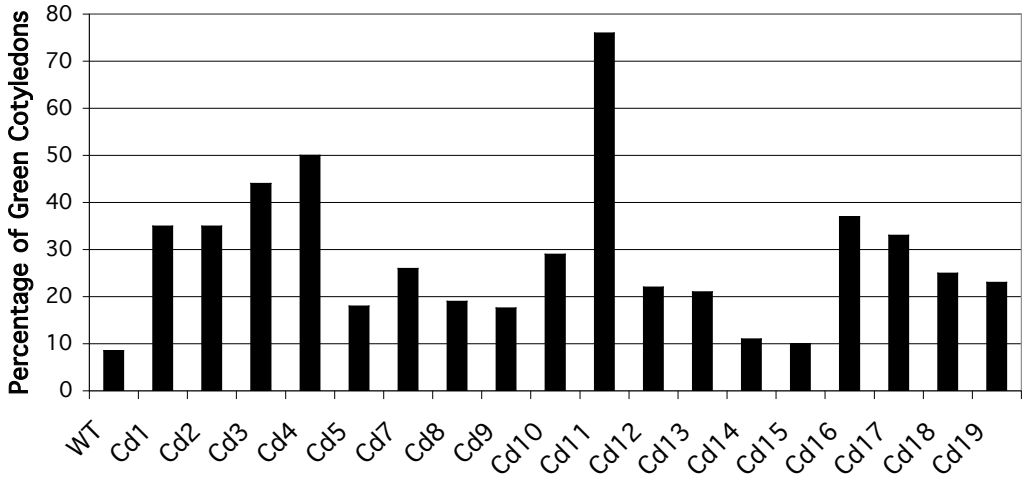


Figure I-2: Cotyledon analysis of EMS generated cadmium tolerant putative mutants. Cotyledon assay of putative cadmium tolerant mutants from EMS mutagenized F3 population. Seedlings were germinated on 0 μM $CdCl_2$ minimal media agar plates for 4 days and transferred to a minimal media plates containing 1.5 mM $CdCl_2$ for an additional 4 days. Y-axis depicts percentage of green cotyledons scored in a population. n= 100-150 seedlings

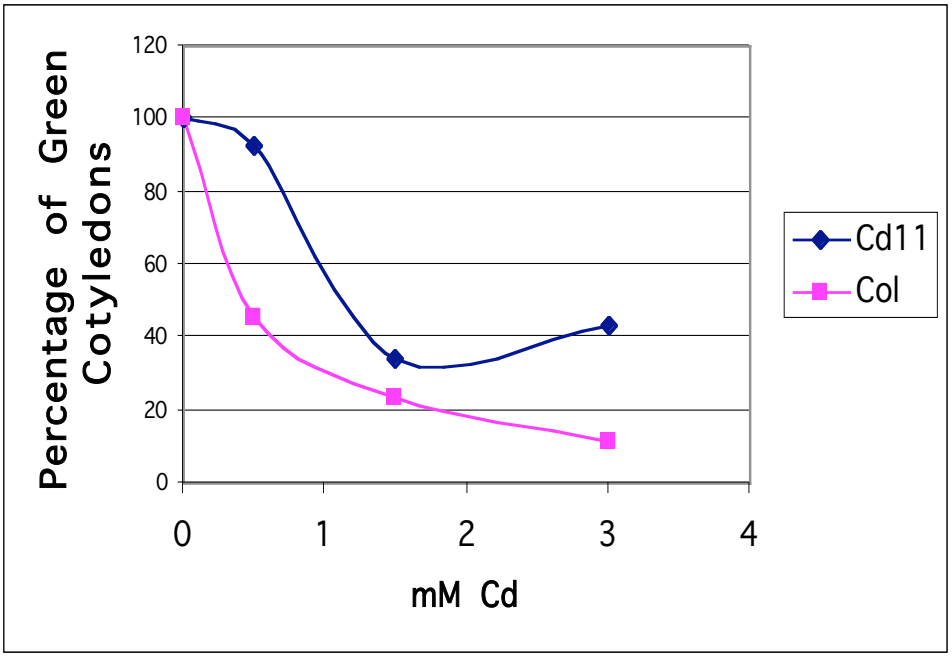


Figure I-3: *Cd11* shoot growth was more tolerant to cadmium in comparison to wildtype.
Cotyledon dose response assay in which seedlings were germinated on MS plates containing 0 μM $CdCl_2$ and for 4 days and transferred to minimal media plates containing the indicated concentrations of cadmium ranging from 0 to 3 mM.

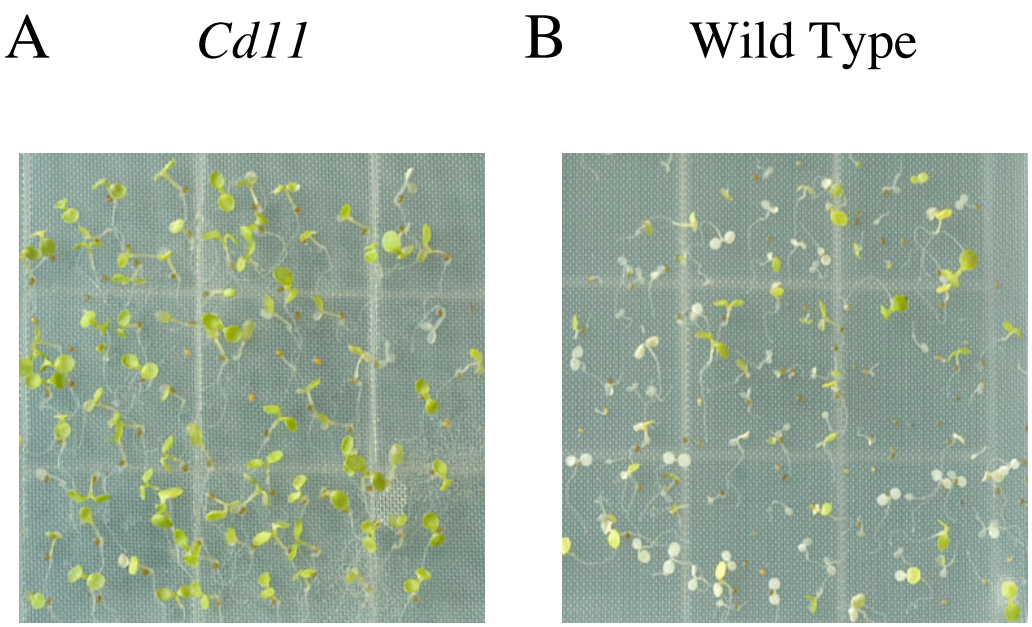


Figure I-4: *Cd11* shoot growth was more tolerant to cadmium in comparison to wild type on 500 μM Cd.
Seedlings grown on MS plates containing 0 μM $CdCl_2$ for four days and transferred to minimal media plates supplemented with 500 μM $CdCl_2$ for an additional four days.
(A) *Cd11*; (B) Wild Type (Col.)

In addition to assaying shoot growth phenotype of *Cd11* to cadmium, root growth analysis was also pursued. Cadmium normally inhibits root growth in *Arabidopsis* (Figure I-5). Seedlings were germinated and grown vertically on minimal media agar plates and transferred to minimal media plates containing 40 μM $CdCl_2$. Root analysis was performed on 40 μM $CdCl_2$ as concentrations above this (60 μM $CdCl_2$) inhibited growth of both *Cd11* and wild type (data not shown). Cadmium concentrations below 40 μM $CdCl_2$ (20 μM $CdCl_2$) did not show a strong distinction between *Cd11* and wild type (data not shown). Root length recorded is a measure of new growth since the transfer to cadmium containing agar plates. *Cd11* shows a longer root length in comparison to wild type (Figure I-5). Therefore in addition to having a greater shoot tolerance, *Cd11* also maintains a great root tolerance in the presence of cadmium in comparison to wild type.

CD11 OVER-ACCUMULATES CADMIUM IN BOTH SHOOT AND ROOT TISSUES

Cadmium accumulation assays are useful for providing insights into the mechanism of cadmium tolerance. Cadmium tolerant mutants that show a lower cadmium accumulation levels in comparison to wild type may be mutated in a transporter that prevents the transport of cadmium into the cytosol. Cadmium tolerant mutants that have either similar or greater levels of cadmium may be altered in their cadmium detoxification or sequestration pathways. Plants used for cadmium accumulation assays were grown under hydroponic conditions and supplemented with

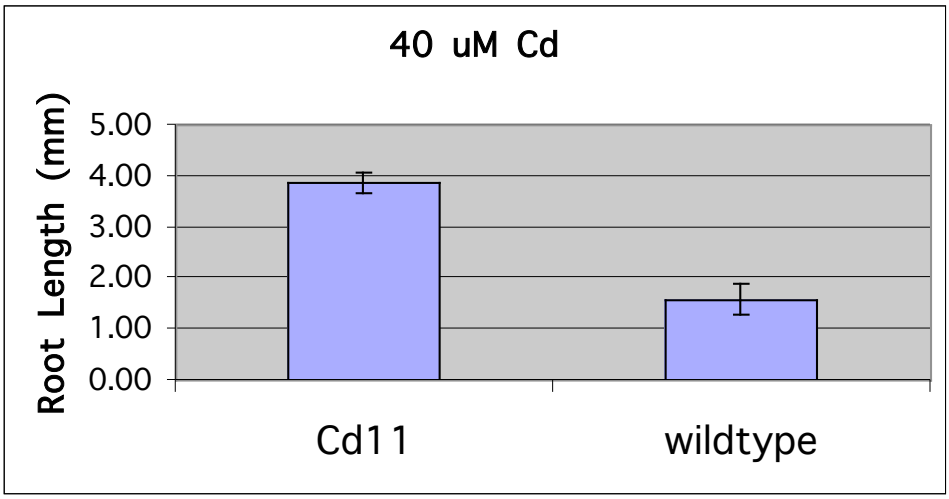


Figure I-5: *Cd11* root growth was more tolerant to cadmium in comparison to wildtype.
Seedlings were germinated on 0 μM $CdCl_2$ minimal media plates for 5 days and transferred to plates containing 40 μM $CdCl_2$ for an additional 3 days. Root length was a measure of new growth since the transfer to 40 μM $CdCl_2$ plates. n= 40 seedlings

20 μM $CdCl_2$ at the bolting stage (approximately 4 weeks). Cadmium accumulation concentrations were assayed using Inductively-Coupled Plasma Optical Emission Spectrometer (ICP-OES). *Cd11* was found to accumulate a higher concentration of cadmium in both root and shoot tissues in comparison to wild type (Figure I-6; n=3 plants). Three other EMS putative cadmium tolerant mutants were also selected for cadmium accumulation analysis (*Cd3, Cd4, Cd16*) and displayed a cadmium accumulation phenotype similar to wild type (data not shown).

SCREENING THROUGH ACTIVATION TAGGED POPULATIONS YIELDED POTENTIAL CADMIUM TOLERANT MUTANTS

The post-germination screen, initially developed for screening EMS mutagenized populations, was also an optimal condition for isolating putative cadmium tolerant mutants from activation tagged lines. A significant percentage of the putative cadmium tolerant mutants from activation tagged lines were able to survive and produce F3 seeds (n=17 of 28 seedlings).

Using the same conditions developed to isolate cadmium tolerant mutants from the EMS mutagenized population, activation tagged seeds were germinated on minimal media plates for 4 days and then transferred with the aid of the porous nylon mesh to minimal media plates containing 1.5 mM $CdCl_2$. Putative mutants that maintained greener and larger cotyledons in comparison to wild type were scored as putative cadmium tolerant mutants. After screening an estimated 58,900-68,050 T-DNA activation tagged lines, 17 putative mutants were isolated.

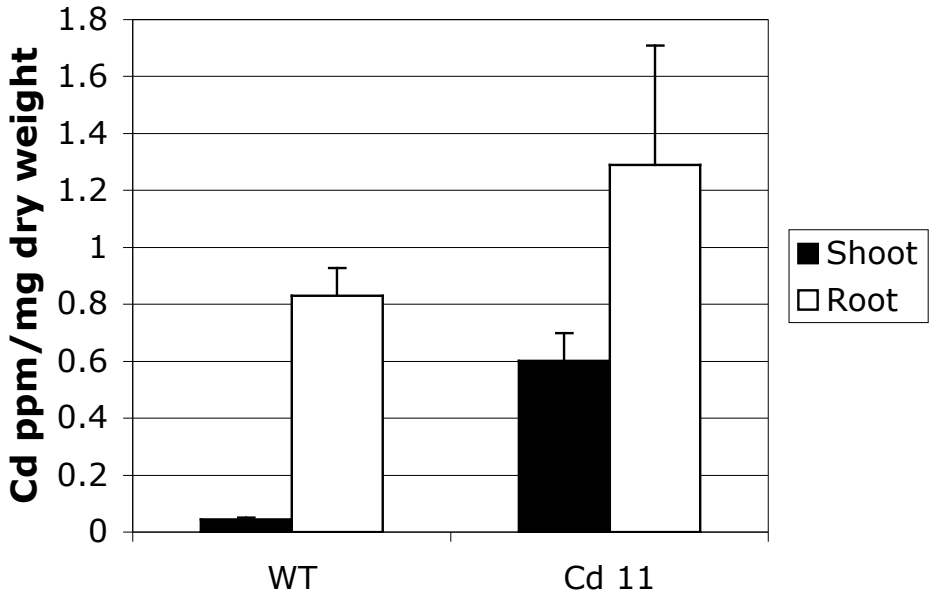


Figure I-6: *Cd11* over-accumulated more cadmium in shoot and root tissues in comparison to wildtype.
Plants were grown under hydroponic conditions with 20 μM $CdCl_2$ added to hydroponic media at bolting stage (approximately four weeks) for 72 hours. Cadmium concentrations were determined by ICP-OES and normalized to dry mass. n=3 plants.

The shoot growth response of the four cadmium tolerant mutants with the highest percentage of green cotyledons was further characterized on a wider range of cadmium concentrations (Figure I-7). *Cd29*, *Cd52*, *Cd54*, and *Cd56* displayed higher concentrations of green cotyledons on plates containing 0, 0.5, 1.5, 3.0 mM of cadmium. All cadmium tolerant mutants, in addition to wild type, were able to germinate and grow normally on control plates not supplemented with cadmium (Figure I-7).

As root growth in *Arabidopsis* was normally inhibited by the presence of cadmium (Figure I-8), the root growth response of the activation tagged mutants was assayed. Root growth in the presence of cadmium of selected activation tagged lines was characterized (Figure I-8). *Cd29* and *Cd56* had longer root lengths in comparison to wild type (Figure I-8). *Cd47*, *Cd54*, and *Cd37* did not display substantially longer roots in the presence of cadmium in comparison to wild type (Figure I-8).

GENETIC ANALYSIS OF ACTIVATION TAGGED LINES

Southern analysis was performed on lines *Cd29*, *Cd37*, *Cd47*, *Cd52*, and *Cd54*. *Cd29* did not contain a T-DNA insertion as determined by the absence of a band, whereas *Cd37* and *Cd47* contained one insertion as indicated by the presence of a single band (data not shown). *Cd52* and *Cd54* either contained insertions with tandem repeats or multiple insertions as indicated by the presence of two bands (data not shown). Unfortunately the southern analysis of *Cd52* and *Cd54* was unclear therefore inconclusive (data not shown).

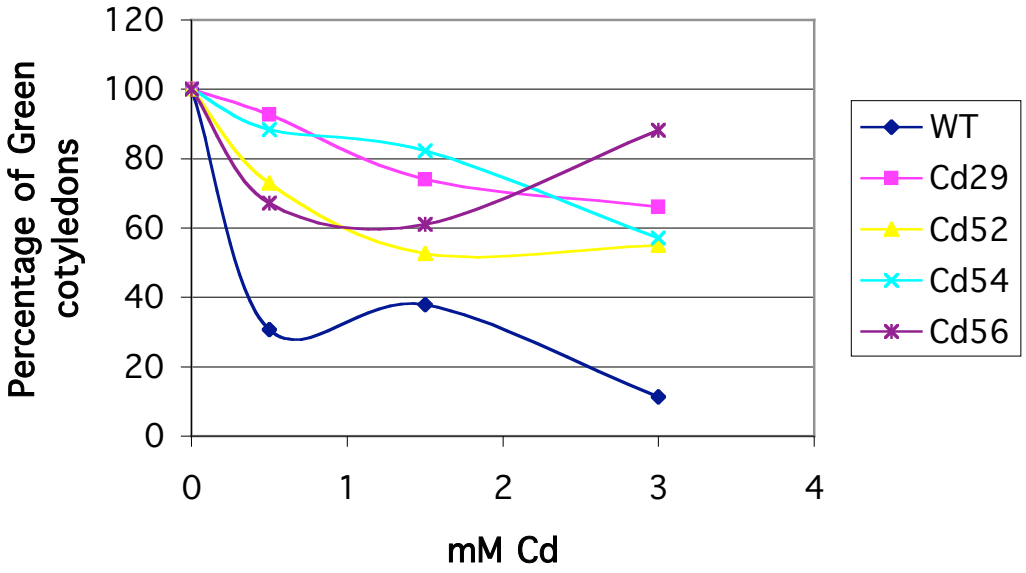


Figure I-7: Cadmium tolerant mutants from selected activation tagged lines showed enhanced shoot growth tolerance in the presence of cadmium in comparison to wild type.
Seeds were germinated on minimal media plates containing 0 μM $CdCl_2$ plates for 4 days on 200 μM nylon mesh and transferred to minimal media plates supplemented with the indicated cadmium concentrations. *Cd29, Cd52, Cd54*, and *Cd56* displayed the highest shoot growth phenotype in comparison to wild type.

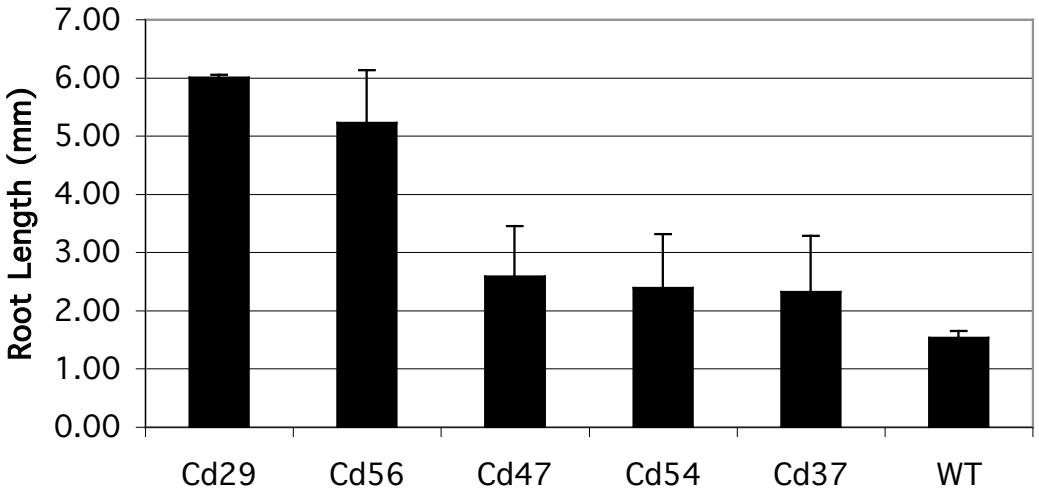


Figure I-8: Cadmium tolerant mutants from selected activation tagged lines displayed more root growth in the presence of cadmium in comparison to wild type.
Seedlings were germinated on minimal media plates containing 0 μM $CdCl_2$ for 5 days and transferred to plates containing 40 μM $CdCl_2$ for an additional 3 days. Root length was a measure of new growth since the transfer to 40 μM $CdCl_2$.

TAIL-PCR was pursued to determine the location of the T-DNA insertion on all activation tagged lines. However, only Cd37 and Cd54 yielded PCR products. Sequencing analysis determined that Cd37 contained a T-DNA insertion in the putative 40s ribosomal protein. Cd54 contained a T-DNA insertion on chromosome 4 between Athb-8 and a putative protein with homology to a GATA transcription factor. Neither the ribosomal protein nor the GATA transcription factor has been characterized to be involved in heavy metal tolerance.

4. DISCUSSION

A post-germination screen was developed to rapidly isolate cadmium tolerant individuals. The post-germination screen, in comparison to a direct germination screen, has been shown to be more successful.

Several different mutagenized populations are available for a mutant screen. EMS is a chemical mutagen that generally produces random point mutations throughout the genome. EMS mutated genes can sometimes be more labor intensive to clone, and therefore only one EMS generated mutant, *Cd11*, was chosen for further analysis. *Cd11* was shown to have greater shoot and root growth in the presence of cadmium. Interestingly, *Cd11* also accumulated higher levels of cadmium in both shoot and root tissues in comparison to wild type. The ability of *Cd11* to accumulate higher levels of cadmium in addition to having greater growth tolerance in the presence of cadmium suggests that *Cd11* contains a mutation in a promoter or gene involved in the cadmium detoxification pathway. Plants may utilize a number of

defense mechanisms in response to cadmium stress such as: (1) immobilization; (2) exclusion; (3) synthesis of phytochelatins; (4) compartmentalization; (5) synthesis of metallothioneins; (6) synthesis of stress proteins; (7) production of stress ethylene (Toppi and Gabbrielli, 1999).

Immobilization is the first barrier against cadmium stress and operates at the root level through the cell wall structure and extracellular carbohydrates. Cadmium ions were found to be mostly bound by pectic sites and hystidyl groups of the cell wall in both root and shoot tissues in the bush bean plants (Leita et al., 1996).

Exclusion involves the regulation of transporters located in the plant cell membrane. Cadmium has been shown to enter through a number of nutrient transporters such as calcium, iron, magnesium, manganese, copper and zinc (Clarkson and Lüttge 1989; Riveta et al., 1997; Clemens et al., 1998; Grotz et al., 1998; Curie et al., 2000; Picard et al., 2000; Thomine et al., 2000; Clemens et al., 2002; Thomine et al., 2003; Papoyan and Kochian 2004). Regulation of nutrient transporters that are permeable to cadmium prevents cadmium ions from entering the cytosol and causing cell damage.

Production of phytochelatins upon heavy metal stress is one of the primary mechanisms plant use to detoxify cadmium ions that have entered the cytosol. The thiol groups of the cysteines and the carboxylic groups of the glutamic acid found in phytochelatins have been shown to bind to heavy metals such as cadmium and prevent cadmium ions from circulating as free Cd^{2+} in the cytosol (Grill et al., 1985; Maitani et al., 1996).

Compartmentalization involves the translocation of cadmium into vacuoles, which limits the amount of free cadmium ions in the cytosol. Phytochelatins bound to heavy metals have been shown to aid in the sequestration of heavy metals into vacuoles (Salt and Rauser, 1995). In addition, free cadmium ions enter the vacuoles by the means of a $Cd^{2+}/2H^{+}$ antiport (Salt and Wagner, 1993; Gries and Wagner, 1998).

Metallothioneins are a group of gene-encoded cysteine rich (approximately 30% cysteine) peptides that generally lack aromatic amino acids (Kägi, 1991). Metallothioneins play a significant role in detoxification of cadmium in mammals, cynaobacteria, and fungi. However in plants metallothioneins are induced and involved in the detoxification primarily of copper (Roosens et al., 2004).

Stress proteins, or heat shock proteins conjugates (hscs) are generally synthesized in response to heavy metal toxicity (Vierling, 1991). The exact function and mechanisms of hscs in relation to heavy metal toxicity are not well known. However, significant levels of hsp70 in *Lycopersicon peruvianum* cell cultures exposed to 1 mM Cd^{2+} were found to be bound to the plasmalemma, mitochondrial membranes, and endoplasmic reticulum (Neumann et al., 1994). Hsp70 has a strong affinity for misfolded proteins and aids in the return of the native confirmation of the protein. This result correlates with the finding in *Saccharomyces cerevisiae* that cadmium causes protein denaturation (Jungmann et al., 1993). Therefore the production of hscs may aid in refolding proteins that have been denatured due to cadmium exposure.

Cadmium has been found to induce the biosynthesis of ethylene, termed stress ethylene, in *Phasiiolus vulgaris* and *Amaranthus lividus* (Adams and Young, 1979; Fuhrer, 1982; Bhattacharjee, 1997). The relationship between the production of ethylene and cadmium stress is not well understood, however when ethylene biosynthesis is inhibited it causes inhibition of phytochelatin synthase activity in carrot cell suspensions and considerable lowering of Cd-induced thiol groups in plants (Sanità di Toppi et al., 1998).

Given the higher accumulation of cadmium in *Cd11* in comparison to wild type (Figure I-6), it seems unlikely that *Cd11* contains a mutation that enhances immobilization and exclusion pathways. Mutants in these two stress mechanisms would exhibit lower levels of cadmium accumulation in comparison to wild type. *Cd11*, however, has higher levels of cadmium accumulation in comparison to wild type (Figure I-6). Therefore it is possible that *Cd11* is mutated in a cadmium stress pathway that deals with the detoxification of cadmium after cadmium enters the cytosol. The mutation of *Cd11* may involve the enhancement of the ability of cadmium to be transported in the vacuole due to a mutated cadmium vacuolar transporter. This hypothesis would take into account the high levels of cadmium observed in both root and shoot tissues (Figure I-6). An enhanced ability to sequester cadmium would also explain the growth tolerance of *Cd11*, as less cadmium would be present in the cystosol. *Cd11* may contain a mutation in a compound that may be either phytochelatins or a compound similar to phytochelatins. This compound may have the ability to bind to cadmium in the cytosol and reduce the concentration of free

Cd^{2+} ions in the cytosol. Phytochelatin assays in *Cd11* would determine if PC levels are altered in *Cd11*. In addition to identifying phytochelatins, fluorescent HPLC coupled to mass spectrometry of plant extracts would also help identify any new thiol containing peptides or proteins in *Cd11* through mass spectrometry analysis. *Cd11* may also be mutated in a heat shock protein conjugate that is involved in cadmium stress response and aids in the refolding of proteins denatured from cadmium exposure. *Cd11* may also be altered in the cadmium-induced stress ethylene response and could possibly enhance down stream signaling of cadmium defense mechanisms such as phytochelatins. Experiments that measure the levels of ethylene in *Cd11* would address whether or not ethylene biosynthesis is altered in *Cd11* in comparison to wild type.

Sequence analysis of the mutated gene would offer insight into the tolerance mechanism of *Cd11*. Identifying the mutation site of EMS generated mutants requires the use of positional markers for mapping and the generation of a significant pool of F1 individuals from a cross between the putative mutant and different ecotype. There are numerous polymorphic markers to distinguish between two different ecotypes in the Cereon database and Monsanto SNPs, which are accessible through the TAIR website. There were a small percentage of individuals in the wild type population that were tolerant to cadmium due to natural genetic variation (Figure I-4). The presence of individuals in a population with altered responses to cadmium makes cloning of heavy metal tolerant mutants difficult due to the interference of false positives. A

larger pool of individuals used for cloning would be required to help mask the effects of false positives.

Activation tagged populations contain random T-DNA insertions. The T-DNA sequence contains four tandem copies of the cauliflower mosaic virus (CaMV) 35S enhancer sequence. The enhancer sequence in the T-DNA may increase the expression of neighboring genes on either side of the T-DNA insertion site, and therefore result in gain-of-function alleles. Activation tagging can also generate loss-of-function alleles through insertional mutagenesis. RT-PCR and/or northern analysis would determine the presence or absence of a full-length mRNA transcript in loss-of-function alleles. As the mutation site will contain a copy of the T-DNA, the site o insertion can be easily identified by either TAIL-PCR or plasmid rescue (Lui et al., 1995; Grant et al, 2003). TAIL-PCR was pursued for several of the activation tagged cadmium tolerant mutants with a low success rate. Plasmid rescue analysis could potentially increase the success rate of determining the T-DNA insertion site. However, one of the drawback of using activation tagged lines is that activation tagged alleles are known to be sometimes unstable (Weigel et al., 2000).

An example of a loss-of-function allele that would confer cadmium tolerance is a loss-of-function allele in a plasma membrane localized transporter that is permeable to cadmium. A mutation of this type would decrease the concentration and toxic effects of cadmium in the cytosol. Although cadmium has been shown to enter the plant through a variety of nutrient transporters, there may be additional transporters that have the ability to transport cadmium. A mutant allele that decreases the transport

of cadmium into the plant would enhance the ability of the mutant plant to grow in the presence of cadmium in comparison to wild type. A potential application of identifying and characterizing a cadmium disrupted transporter would be to engineer crop plants to accumulate less cadmium, and thereby decreasing the amount of cadmium in human consumption. Preliminary data on cadmium accumulation in EMS lines showed that most mutants accumulated either the similar or greater concentrations of cadmium in comparison to wild type (Figure I-6; data not shown). This suggests that mutations in cadmium transporters may be difficult to detect due to the ability of cadmium to enter through a variety of nutrient transporters.

Gain-of-function alleles isolated from a cadmium tolerance screen of activation tagged lines could potentially affect genes involved in cadmium detoxification mechanisms. Examples of cadmium detoxification mechanisms include the biochemical breakdown of toxic compounds to less toxic compounds or enhanced sequestration of cadmium compounds into vacuoles as a means of protecting the cytoplasm from heavy metal toxicity.

The post-germination screen has been shown to have the ability to successfully isolate cadmium tolerant mutants. EMS generated *Cd11* has shown to have shoot and root growth tolerance in comparison to wild type. In addition, ICP-OES analysis has shown that *Cd11* accumulates higher concentrations of cadmium in both shoot and root tissues in comparison to wild type. Genetical and phytochelatin analysis have yet to be pursued in *Cd11* and would offer insight into the mechanism its cadmium tolerance. As the genome of many organisms, including *Arabidopsis*, has been

sequenced and the function of many genes characterized, a homology search of the mutated *Cd11* gene would aid in determining gene function. Cadmium tolerant mutants from activation tagged lines have also been isolated, however further work such as cadmium accumulation profile, plasmid rescue to determine T-DNA insertion sites, dominant and recessive genetic analysis, and phytochelatin analysis, still need to be performed.

II.

Isolation and Characterization of *atpcs2-1* and *cad1-3 atpcs2-1*

1. Abstract

Phytochelatins (PCs) are small thiolate peptides that have been shown to confer heavy metal tolerance in plants, fungi, and nematodes. Phytochelatins are synthesized post-translationally and catalyzed by the enzyme phytochelatin synthase (PCS). *Arabidopsis thaliana* has two functional *AtPCS* genes, *AtPCS1* and *AtPCS2*. A loss-of-function *AtPCS1* mutant, *cad1*, has been isolated and characterized. *cad1* was observed not to produce detectable phytochelatins and is sensitive to heavy metal stress in comparison to wild type. The sequencing of the *Arabidopsis* genome lead to the identification of a gene (*AtPCS2*) with an 84% coding region identity to *AtPCS1*. The function of *AtPCS2* is unclear, because the *AtPCS1* loss-of-function allele *cad1-3* is sensitive to heavy metals and does not produce detectable phytochelatins. To date there have been no publications on the characterization of a loss-of-function allele in the *AtPCS2* gene. Here we present the isolation and characterization of an *AtPCS2* T-DNA insertion loss-of-function allele (*atpcs2-1*) and the generation and characterization of a loss-of-function *AtPCS* double mutant *cad1-atpcs2-1*. *atpcs2-1* did not appear to have any significant difference in regards to shoot and root growth response to cadmium in comparison to wild type. In addition, the cadmium accumulation and distribution pattern of *atpcs2-1* in comparison to wild type did not show any significant difference. Phytochelatin analysis showed the presence of PC2, PC3, and PC4 in *atpcs2-1*. The *cad1-3 atpcs2-1* double mutant did not have any significant difference in shoot and root growth response in the presence of cadmium in comparison to the single mutant *cad1-3*. In addition, the cadmium accumulation and

distribution patterns were similar between *cad1-3 atpcs2-1* and *cad1-3*, in which both mutants showed over-accumulation of cadmium in root tissues and under-accumulation of cadmium in shoot tissues in comparison to wild type. Phytochelatin analysis showed that both *cad1-3 atpcs2-1* and *cad1-3* lacked detectable phytochelatins.

2. Introduction

The primary mechanism plants employ to tolerate heavy metal toxicity is the production of small thiolate peptides called phytochelatins (PCs). PCs have the ability to bind a variety of heavy metals (Kondo, 1984; Grill et al., 1985). Phytochelatins were first discovered in the *Schizosaccharomyces pombe* and termed cadystins (Kondo et al., 1984). PCs have since been discovered in all plant species investigated, marine diatoms, fungus, and nematodes (Vatamanuik, et al., 2001; Clemens et al., 2001; Cobbett et al., 2002). PCs are induced in a wide range of plant species by the oxy-anions arsenate [$As^{(V)}$] and selenate as well as by a range of cations such as Ag^{+}, Cd^{2+}, Cu^{+}, Hg^{2+}, and Pb^{2+} (Grill et al., 1985).

Phytochelatins, which have the chemical structure $(\gamma Glu\text{-}Cys)_n$-Gly where n=2-11, are produced post-translationally by the dipeptidyltransferase enzyme phytochelatin synthase (PCS) with reduced glutathione as a precursor (Grill et al., 1989; Ha et al., 1999; Clemens et al., 1999;Vatamanuik et al., 1999; Vatamanuik et al., 2004; Figure II-1). Phytochelatins form stable complexes with heavy metals in the cytosol, and these complexes are sequestered into the vacuole as a detoxification

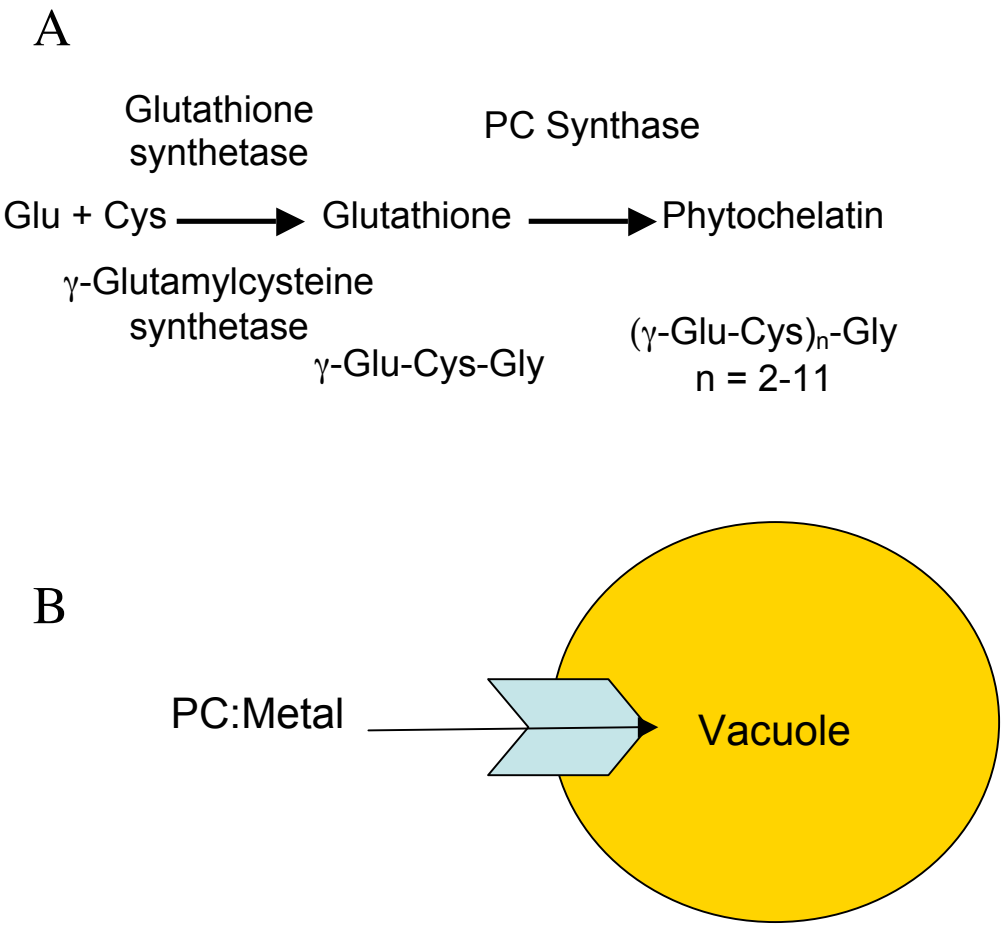


Figure II-1: Diagram illustrating the biochemical synthesis pathway of phytochelatins and sequesteration of heavy metals into vacuoles through phytochelatins. (A) Biochemical pathway for the post-translational synthesis of phytochelatins with reduced glutathione as a precusor. Enzymes are located directly above and below arrows. (B) Phytochelatins bind to heavy metals in the cytsol of cells and aid in the sequestration of heavy metals into a vacuole.

mechanism (Cobbett 2000a; 2000b; Grill et al., 1985; Zenk et al., 1996). In the yeast, *Schizosaccharomyces pombe*, complexes of heavy metals bound to PCs are transported across the tonoplast and sequestered in vacuoles by means of the ATP binding cassette transporter HMT1 (Ortiz, et al 1995). Recently a HMT1 homolog in *Caenorhabditis elegans* was identified (*CeHMT-1*), and demonstrated to be required for cadmium tolerance (Vatamaniuk et al., 2005). There is no direct homolog of HMT1 in the *Arabidopsis* genome, and to date the identification of the vacuolar PC transporter in plants has not been reported.

The Arabidopsis genome has two PCS genes: *AtPCS1* and *AtPCS2*. *AtPCS1* (AT5G44070) is located on chromosome 5 and predicted to encode for a 55-kD protein consisting of 485 amino acids. *AtPCS1* mRNA is present in the absence of heavy metals in all tissue types, but is not activated to induce the production of phytochelatins without the presence of heavy metals (Ha et al., 1999; Clemens et al., 1999; Vatamanuik et al., 1999). Activation of the PCS enzyme occurs when heavy metals bind and block the thiol groups located near the N-terminal domain (Vatamanuik et al., 2000). Cadmium is one of the strongest heavy metal inducers of phytochelatin production. A loss-of-function mutation in *AtPCS1* (*cad1)* has been isolated and well characterized in *Arabidopsis*. The mutant allele *cad1-3* is sensitive to heavy metals in comparison to wild type and does not produce detectable phytochelatins (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003). In addition, heterologous expression of phytochelatin synthase from *Arabidopsis*

(*AtPCS1*), *Schizosaccharomyces pombe* (*SpPCS1*), and wheat (*TaPCS1)* in *Saccharomyces cerevisiae*, which lacks a native *PCS* gene and phytochelatins, have resulted in metal inducible PC formation (Clemens, et al., 1999). *AtPCS1* has been found to be expressed in all analyzed tissue types including roots, flowers, rosette leaves, and stems (Cazalé and Clemens, 2001).

The Arabidopsis genome has two PCS genes: *AtPCS1* and *AtPCS2*. *AtPCS1* (AT5G44070) is located on chromosome 5 and predicted to encode for a 55-kD protein consisting of 485 amino acids. *AtPCS1* mRNA is present in the absence of heavy metals in all tissue types, but is not activated to induce the production of phytochelatins without the presence of heavy metals (Ha et al., 1999; Clemens et al., 1999; Vatamanuik et al., 1999). Activation of the PCS enzyme occurs when heavy metals bind and block the thiol groups located near the N-terminal domain (Vatamanuik et al., 2000). Cadmium is one of the strongest heavy metal inducers of phytochelatin production. A loss-of-function mutation in *AtPCS1* (*cad1)* has been isolated and well characterized in *Arabidopsis*. The mutant allele *cad1-3* is sensitive to heavy metals in comparison to wild type and does not produce detectable phytochelatins (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003). In addition, heterologous expression of phytochelatin synthase from *Arabidopsis* (*AtPCS1*), *Schizosaccharomyces pombe* (*SpPCS1*), and wheat (*TaPCS1)* in *Saccharomyces cerevisiae*, which lacks a native *PCS* gene and phytochelatins, have resulted in metal inducible PC formation (Clemens, et al., 1999). *AtPCS1* has been

found to be expressed in all analyzed tissue types including roots, flowers, rosette leaves, and stems (Cazalé and Clemens, 2001).

Considering that mutants deficient in *AtPCS1* (*cad1)* were found to be sensitive to heavy metals and lacked detectable PCs, it was surprising to find a second *PCS* homolog (*AtPCS2)* after the sequencing of the *Arabidopsis* genome. *AtPCS2* (AT1G03980) is located on chromosome 1 and shares an 84% coding region identity with *AtPCS1* (Cazalé and Clemens, 2001). Northern and western blot analysis has shown that *AtPCS2* mRNA and protein expression is lower in comparison to AtPCS1, and *AtPCS2* does not appear to have tissue specific expression (Cazalé and Clemens, 2001). In comparison to *AtPCS1*, *AtPCS2* has intron sequences that have diverged, and hence *AtPCS2* does not appear to be the result of a recent duplication event in the *Arabidopsis* genome (*Arabidopsis* Genome initiative, 2000). *AtPCS2* has been functionally characterized in yeast and confered a small degree of heavy metal tolerance in yeast and synthesized PCs in the presence of cadmium (Cazalé and Clemens, 2001). One of the characteristic biochemical properties of a phytochelatin synthase is the ability to be activated by a variety of metals and metalloids. *AtPCS2* is similar to *AtPCS1* in that it could be activated by cadmium, arsenate, zinc, magnesium, and nickel (Cazalé and Clemens, 2001). However, unlike *AtPCS1*, *AtPCS2* could not be induced by the presence of copper (Cazalé and Clemens, 2001). In RT-PCR analysis, *AtPCS2* was expressed in leaves, stems, flowers, and roots at levels lower than that of *AtPCS1* (Cazalé and Clemens, 2001). The low expression of *AtPCS2* correlates with the reports that the promoter of *AtPCS2* has low activity. In

addition, *AtPCS2* has low efficiency in the translation of its mRNA, which would explain the absence of detectable phytochelatins in *cad1* (*AtPCS1*) mutants (Lee et al., 2005).

To gain further insight into the function of *AtPCS2*, we isolated and characterized a loss-of-function allele in *AtPCS2* (*atpcs2-1*). An *AtPCS* loss-of-function allele double mutant (*cad1-3 atpcs2-1*) was then generated by crossing *cad1-3* with *atpcs2-1* and identified through PCR genotyping. The *AtPCS2* loss-of-function allele (*atpcs2-1*) was isolated through PCR-based screening of the Wisconsin T-DNA insertion collection. These T-DNA tagged lines were generated by the insertion of a T-DNA when a portion of the *Agrobacterium* Ti plasmid is transferred into plant cells during *Agrobacterium* mediated plant transformation. One of the advantages of screening through a large pool of mutants is the increased chance of finding one or more alleles containing a T-DNA insertion in your gene of interest (Krysan et al., 1999). Analysis of 1000 sequenced T-DNA tags revealed that most T-DNA insertions were in regions of high gene density and that nearly 50% of T-DNA insertions were in coding sequence of 5' regulatory sequence (Szabados et al 2002). However, one of the drawbacks of this method is the high number of chromosomal rearrangements that may make mutant analysis difficult (Tax and Vernon, 2001).

3. Methods

ISOLATION AND SCREENING OF T-DNA INSERTION IN *ATPCS2*

A T-DNA loss-of-function allele in *PCS2* was isolated through screening the

University of Wisconsin Knockout Facilities generated T-DNA pools (give website). *Arabidopsis thaliana* (Ws background) was transformed with a derivative of the T-DNA vector pD991:Pd991-AP3 (Krysan, et al., 1999) Screening the T-DNA populations was performed as described in http://www.biotech.wisc.edu/Arabidopsis/Index2.asp. Plants homozygous for the T-DNA insertion in *AtPCS2* were identified from segregating populations via PCR analysis using a primer specific to the T-DNA left border (JL-202: 5'CATTTTATAATAACGCTGCGGACATCTAC3') and a primer approximately 2.3 kb downstream from the start site of *AtPCS2* (3' Cdr2-N: 5'CTGACCACGTCTGTGGAGGTAAAGCCAAC3'). Nested PCR was performed to confirm T-DNA insertion using primers JL-270: TTTCTCCATATTGACCATC ATACTCATTG and Cdr2-N. Locations of insertions were confirmed by sequencing the flanking DNA (Retrogen, San Diego).

PLANT MATERIAL, GROWTH CONDITIONS, AND METAL STRESS TREATMENTS

For shoot growth analysis Arabidopsis seedlings were grown horizontally on one-quarter-strength Murashige and Skoog (MS) basal medium (Sigma Aldrich), 1 mM MES, 1% agar for a period of four days on nylon mesh with a 200 μM pore size (Spectrum Labs, TX). Seedlings and nylon mesh were then transferred to MS plates supplemented with 1 mM $CdCl_2$ for an additional four days. Seedlings used for shoot growth analyses were grown horizontally under 16/8-h day/night period at 23^0C.

For analysis of Cd^{2+}-dependent root growth, seeds were sterilized and plated on plates containing one-quarter strength MS medium, 1 mM MES, 1% Agar Type A (Sigma Aldrich), acclimated with cold treatment at 4 ºC for 48 hours, and grown vertically under 24 hour light growth room conditions for five days. Seedlings were then transferred to quarter strength MS, 1 mM MES, 1% Agar plates containing 20 μM $CdCl_2$ for an additional 72 hours vertical growth.

Plants used for Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) and Fluorescence HPLC analysis were grown under hydroponic conditions with 80 mL of medium as described (Arteca and Arteca, 1998) with minor modifications: Plants were germinated in Petri dishes and grown vertically for the first four days and subsequently moved to a horizontal position for an additional two days to bend the hypocotyl to aid in successful transplantation onto the hydroponic sponge. Plants were grown under hydroponic conditions at 24ºC under a 16/8-h day/night period and hydroponic media were replaced every 3-4 days. After reaching the bolting stage (approximately 4 week old plants) hydroponic media (50 mL) were replaced with the same media to which 20 μM $CdCl_2$ was added for a period of four days for cadmium accumulation analyses, whereas the hydroponic media containing 20 μM $CdCl_2$ was added for a period of three days for induction of PCs. Shoot and root tissues were rinsed three times in deionized water for fluorescence HPLC coupled to mass spectrometry analyses.

ICP-OES

To measure metal accumulation, plants were grown under hydroponic conditions in liquid media (Arteca and Arteca, 2000) until bolting stage (approximately 4 weeks). Shoots were rinsed 3 times in deionized water. For ICP-OES analyses of root tissue, roots were rinsed in deionized water, washed in 100 mL of 100 mM $CaCl_2$ on an orbital shaker (Bellco Glass, Inc., NJ) for 5 minutes at approximately 135 rpm (speed setting #4), and then washed in 100 mL of deionized water on the orbital shaker (speed setting #4) for an additional 3 minutes. Shoot tissues were separated immediately below the cotyledons and above the hydroponic sponge. Root tissues were separated below the hydroponic sponge, approximately 3 cm below cotyledons. Both shoot and root tissues were dried at 60^{o}C overnight. The dry weight was recorded, and then the tissues were digested in 70% trace metal grade nitric acid (Fisher Scientific) overnight. Samples were then boiled for 30 minutes to ensure complete digestion and diluted to a final concentration of 5% nitric acid with deionized water.

4. Results

ATPCS2-1 IS A LOSS-OF-FUNCTION ALLELE

Reverse genetics is a powerful tool for elucidating the function of specific genes with known sequences. Screening for T-DNA insertions within a specific gene using PCR-based methods is a relatively quick and easy method for obtaining loss-of-function alleles. The *atpcs2-1* loss-of-function allele was isolated from screening

through the Wisconsin T-DNA insertion lines. Sequencing analysis has determined that a single T-DNA is inserted within the sixth intron of *AtPCS2*.

To determine whether *atpcs2-1* was a loss-of-function allele, RT-PCR analysis using primers to both the promoter region and the 5' terminus of *AtPCS2* has demonstrated the absence of the full length *AtPCS2* transcript in the *atpcs2-1* mutant. (Figure II-2). Three independent plants (Figure II-2; P-1, P-2, P-3) were analyzed and confirmed not to contain the full length *AtPCS2* transcript. Wild type (Ws ecotype) served as a positive control. PCR amplification of Elongation Factor 1α (EF1α) was used as a loading control and demonstrated equal loading of all four lines.

SHOOTS AND ROOTS OF *ATPCS2-1* SHOWED SIMILAR GROWTH RESPONSE TO CADMIUM AS WILD TYPE

Shoots of wild type plants are normally sensitive to the presence of cadmium and undergo chlorosis. Cotyledons are the first two leaves to emerge from the seed coat, and in wild type plants the cotyledons become chlorotic in the presence of high concentrations of cadmium. Seedlings were germinated on a porous nylon mesh placed on top of minimal media MS agar plates not containing cadmium for a period of four days. Seedlings were then transferred with the aid of the nylon mesh to plates containing 1 mM cadmium. Shoot growth analysis demonstrated that *atpcs2-1* is tolerant to 1 mM Cd in comparison to *cad1-3*, as *atpcs2-1* (Figure II-3; 37%) was found to have a similar percentage of green cotyledons as wild type (Figure II-3;

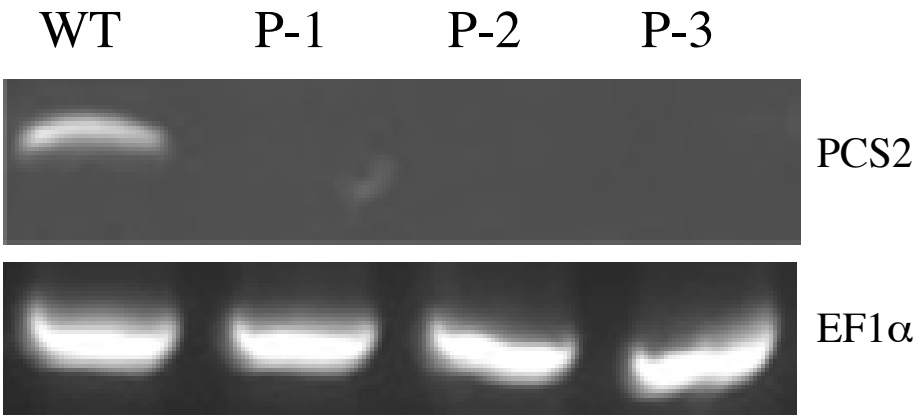


Figure II-2: *atpcs2-1* is a loss-of-function allele.

RT-PCR performed on three individual *atpcs2-1* plants (P-1, P-2, P-3) with primers for the At*PCS2* 5'UTR region and the At*PCS2* N-terminus. Wild type (WT; Ws ecotype) served as a positive control (WT). Elongation Factor (EF1α) primers used as a loading control.

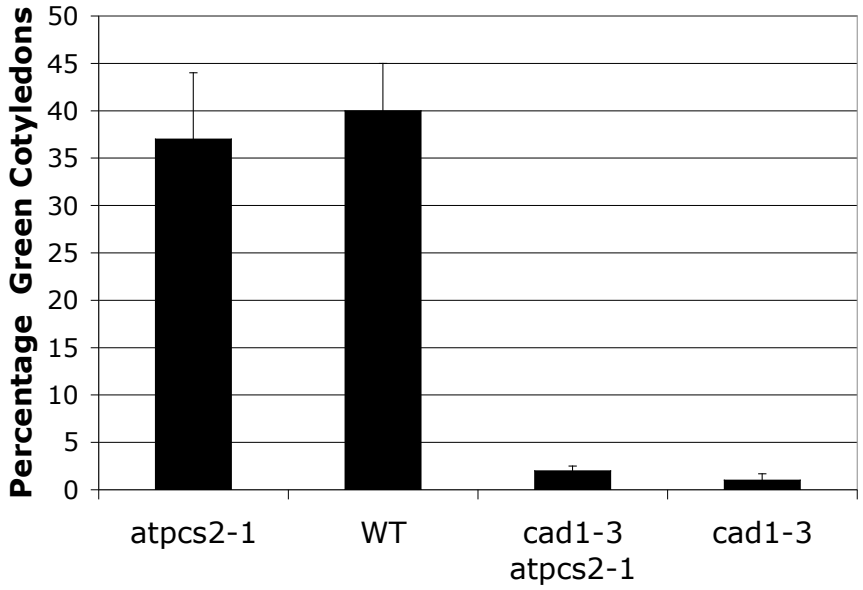


Figure II-3: *atpcs2-1* shoots show similar shoot growth response to cadmium as wild type.
The Y-axis represents the percentage of green cotyledons in a population. *atpcs2-1* had a similar cadmium tolerance phenotype on 1 mM $CdCl_2$ in comparison to wild type (WT). Seedlings were germinated on 0 mM $CdCl_2$ for four days and transferred to plates containing 1 mM $CdCl_2$ for an additional four days. *cad1-3 atpcs2-1* demonstrated similar cadmium sensitivity as the single mutant *cad1-3*.

40%). The similar percentages of green cotyledons in *atpcs2-1* compared with wild type suggests that a functional *AtPCS1* gene overcomes the loss of *AtPCS2*. *cad1-3 atpcs2-1*, in comparison to wild type, shows shoot growth sensitivity in the presence of cadmium (Figure II-3). The similar percentages of green cotyledons in *cad1-3* and *cad1-3 atpcs2-1* may be due to the lack of a functional *AtPCS1*.

In addition to characterizing shoot growth, we also examined the root growth of *atpcs2-1* and *cad1-3 atpcs2-1*, because root growth is also sensitive to cadmium in wild type plants. Root growth analysis showed that *atpcs2-1* is tolerant to 20 μM cadmium in comparison to the *AtPCS1* loss of function allele cad1-*3* (Figure II-4). However, *atpcs2-1* root growth on cadmium compared to wild type (Ws ecotype) did not show a significant difference. This may be due to the presence of a functional *AtPCS1* gene in both wild type and *atpcs2-1*. The double *AtPCS* mutant, *cad1-3 atpcs2-1,* shows root sensitivity on cadmium in comparison to wild type (Ws x Col F2; Figure II-4). However, the root length of *cad1-3 atpcs2-1* was shown to be as sensitive to the presence of cadmium as the single *AtPCS1* mutant *cad1-3* (Figure II-4). Both *cad1-3 atpcs2-1* and *atpcs2-1* lack a functional *AtPCS1* and hence are sensitive to cadmium stress.

CADMIUM ACCUMULATION, CONCENTRATION, AND TISSUE DISTRIBUTION OF *ATPCS2-1* IS SIMILAR TO WILD TYPE

Plants were grown under hydroponic conditions and supplemented with 20 μM cadmium at the bolting stage (approximately 4 weeks). Cadmium accumulation was

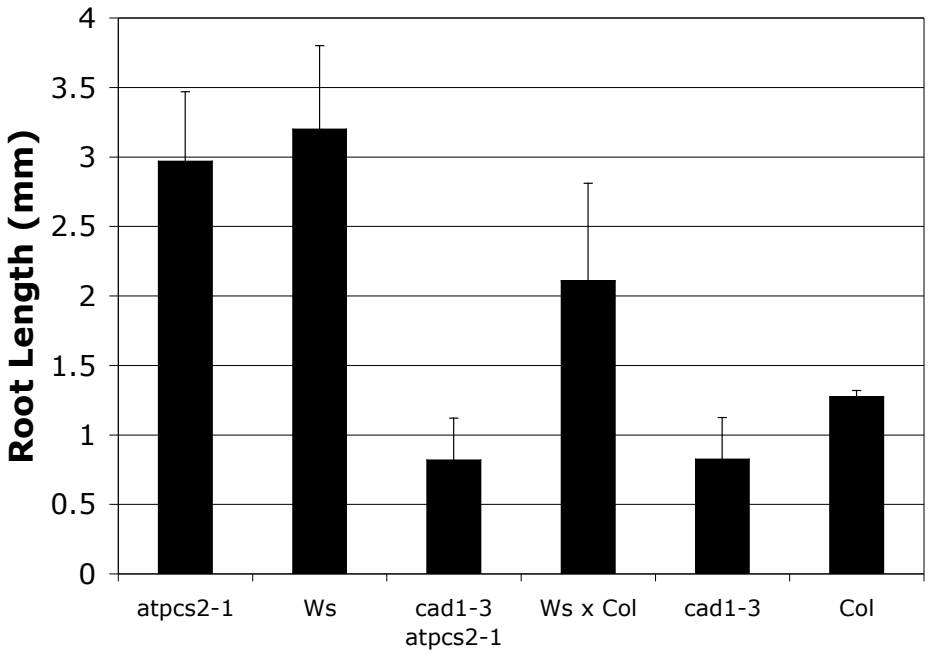


Figure II-4: Roots of *atpcs2-1* showed similar growth response to cadmium as wild type.
Y-axis represents root length in millimeters (mm). Seedling were grown vertically on minimal media plates for 5 days and transferred to minimal media plates supplemented with 20 μM $CdCl_2$ for an additional 3 days. Root length is a measure of new growth since the transfer to 20 μ M $CdCl_2$.

assayed by using ICP-OES and normalized to dry weight. Cadmium accumulation in *atpcs2-1* showed a similar distribution pattern and concentration to wild type (Ws ecotype; Figure II-5). Both *atpcs2-1* and wild type showed more cadmium accumulation in root tissues compared to shoot tissues. In contrast, both the double mutant *cad1-3 atpcs2-1* and the single mutant*cad1-3* over accumulated cadmium in root tissues (Figure II-5). Phytochelatins have recently been implicated in aiding long distance transport of cadmium from roots to shoots (Gong et al., 2003) and the absence of a functional *AtPCS1* in *cad1-3* and *cad1-3 atpcs2-1* may explain the over accumulation of cadmium in root tissues.

PHYTOCHELATINS ARE SYNTHESIZED IN *ATPCS2-1*

Fluorescence HPLC analyses of monobromobimane labeled plant extracts were performed to analyze the levels of PCs (Figure II-6). Plants were grown under hydroponic conditions and the hydroponic media was supplemented with 20 μM cadmium at the bolting stage (approximately 4 weeks) to induce the production of phytochelatins. PC2, PC3, and PC4 peaks were identified using synthesized PC standards and are shown in panels I and J of Figure II-6. *cad1-3* plants did not show detectable phytochelatins (Figure II-6, A and B; n=14 of 14 plants), which confirms published reports on *cad1-3* (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003). PC2, PC3, and PC4 were clearly detected in positive control wildtype shoot and root tissues of 4 week old plants exposed to cadmium (Figure II-6, G and H; n=6 of 6 plants). As *atpcs2-1* maintains a functional *AtPCS1* which, has the ability to

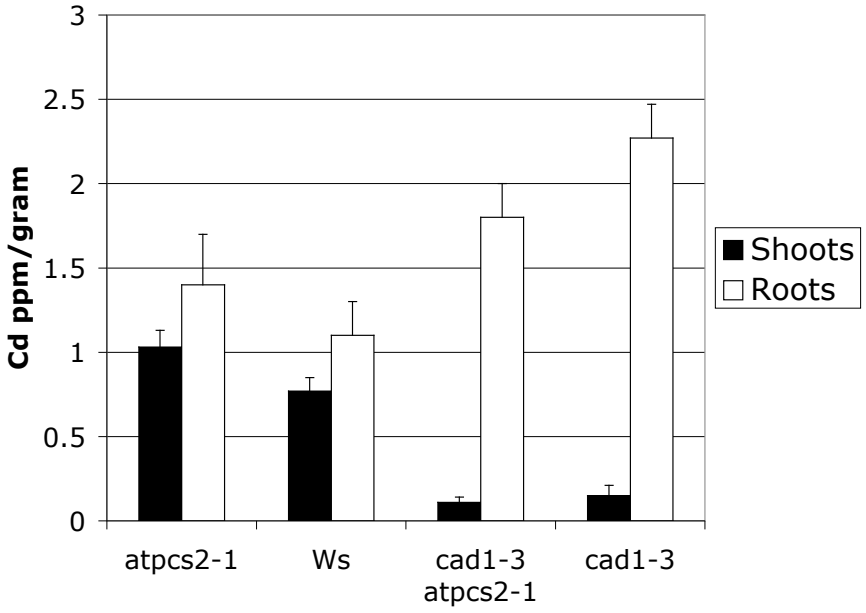


Figure II-5: *atpcs2-1* has similar cadmium accumulation and distribution pattern as wild type. *cad1-3 atpcs2-1* over accumulates cadmium in root tissues in comparison to wild type.

Y-axis shows cadmium parts per million (Cd ppm) and is normalized to dry weight (grams). *cad1-3 atpcs2-1* had higher cadmium accumulation in root tissues than in shoot tissues and has a distribution pattern similar to wild type (Ws). *cad1-3 atpcs2-1* over-accumulated cadmium in root tissues compared to wild type (Ws), but demonstrated a similar cadmium accumulation and distribution pattern as *cad1-3*.catalyze the synthesis of phytochelatins, *atpcs2-1* also showed the presence of PC2, PC3, and PC4 peaks (Figure II-6, E and D; n=12 of 12 plants), whereas *cad1-3 atpcs2-1* did not demonstrate detectable phytochelatins (Figure II-6; C and D; n=10 of 10 plants).

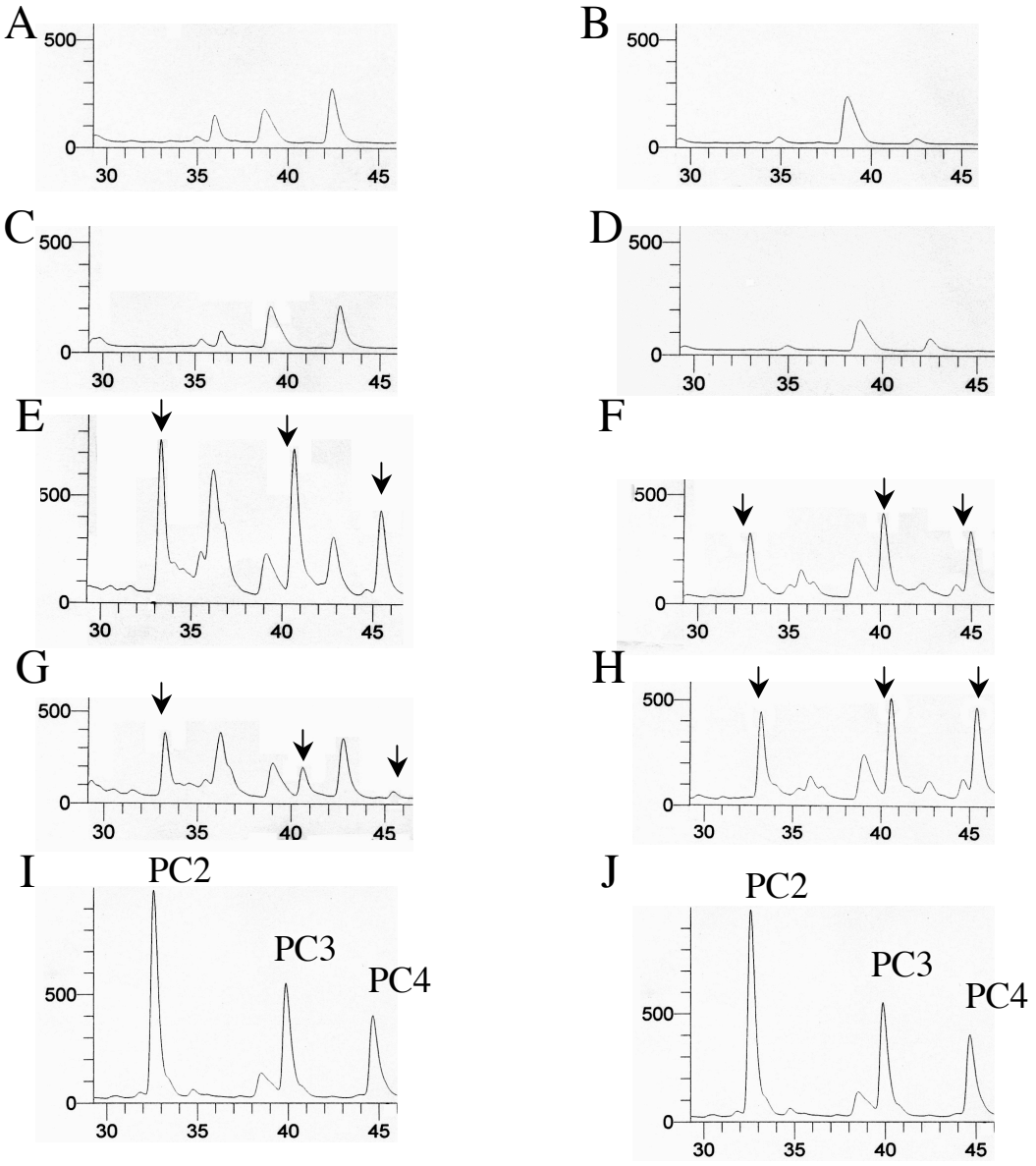


Figure II-6: *atpcs2-1* has produces phytochelatins and *cad1-3 atpcs2-1* has no detectable phytochelatins.
All peaks corresponding to phytochelatins are indicated with arrows. Shoot tissue samples are shown in the left hand column and root tissue samples are shown in the right hand column. (A, B) *cad1-3* as a negative control; (C, D) *cad1-3 atpcs2-1*; (E, F) *atpcs2-1* has PC2, PC3, and PC4; (G, H) Wild Type (Ws) serving as a positive control with PC2, PC3, and PC4; (H, I) Synthesized PC2, PC3, and PC4 standards.

catalyze the synthesis of phytochelatins, *atpcs2-1* also showed the presence of PC2, PC3, and PC4 peaks (Figure II-6, E and D; n=12 of 12 plants), whereas *cad1-3 atpcs2-1* did not demonstrate detectable phytochelatins (Figure II-6; C and D; n=10 of 10 plants).

5. Discussion

AtPCS2 has been demonstrated to have the ability to confer heavy metal tolerance and produce functional phytochelatins when expressed in *S. pombe* cells lacking a functional *SpPCS1* (Cazalé and Clemens, 2001). It had been previously suggested that *AtPCS2* may be expressed only in specific cell types, which may explain the absence of detectable phytochelatins in *cad1* mutants if the cell types expressing *AtPCS2* are few in comparison to cells expressing *AtPCS1* (Cazalé and Clemens, 2001). However, it was recently demonstrated that *AtPCS2* seems to be expressed at a very low intensity in both root and shoot tissues (Lee et al., 2005). Analysis of the promoter of *AtPCS2* has shown the promoter to be significantly weaker in comparison to *AtPCS1* (Lee et al., 2005). However, when the *AtPCS2* gene was expressed under the control of strong promoters and transformed into *cad1-3* mutants, it failed to completely complement the cadmium sensitivity of *cad1-3* (Lee et al., 2005). Therefore *AtPCS2* appears to have low efficiency of mRNA translation in addition to having a weak promoter, which explains why there are no detectable phytochelatins in the *AtPCS1* loss-of-function mutant *cad1-3*.

We have isolated a loss-of-function allele in *AtPCS2*, *atpcs2-1*, to further understand the role of *AtPCS2* in heavy metal detoxification. There was no significant difference between wild type (Ws ecotype) and the loss-of-function *atpcs2-1* in regards to shoot (Figure II-3) or root growth (Figure II-4) in the presence of cadmium, cadmium accumulation (Figure II-6), and phytochelatin levels in the plants (Figure II-6). These results confirm published conclusions that *AtPCS2* plays a minor role in comparison to *AtPCS1* in heavy metal detoxification (Cazalé and Clemens, 2001; Lee et al., 2005). Both wild type and *atpcs2-1* maintain a functional *AtPCS1* and therefore are not as sensitive to cadmium stress in comparison to the *AtPCS1* loss-of-function *cad1-3*.

We have isolated a double *AtPCS* mutant, *cad1-3 atpcs2-1*, for characterization. There was no significant difference between the single *AtPCS1* loss-of-function allele *cad1-3* and the *AtPCS* double loss-of-function mutant *cad1-3 atpcs2-1* in regards to shoot (Figure II-3) and root growth (Figure II-4) in the presence of cadmium, cadmium accumulation (Figure II-5), and phytochelatin levels (Figure II-6). The single *AtPCS1* loss-of-function mutant, *cad1-3*, is sensitive to heavy metal stress (Cobbett et al., 1998; Howden et al., 1995; Gong et al., 2003). It was therefore not unexpected to discover that the double *AtPCS* mutant, *cad1-3 atpcs2-1*, displays similar sensitivity to cadmium stress.

Therefore the phenotype of *atpcs2-1* did not appear to be easily discernable given the assays performed. The conclusion that *AtPCS2* does not play a major role in heavy metal detoxification compared to *AtPCS1* is consistent given the results from

our characterization of *atpcs2-1* and published data. All assays performed to elucidate the function of *AtPCS2* have been to determine its role in heavy metal detoxification, and it is possible that *AtPCS2* may also have another function independent of catalyzing the production of phytochelatins. It is possible that *AtPCS2* acts as a signaling molecule as signaling molecules do not need to be expressed at high levels in order to be effective. This would explain the low expression levels reported for *AtPCS2* (Lee, et al., 2005). *AtPCS2* could act as a signal to upregulate heavy metal tolerance pathways. Quantative PCR could be performed on genes regulated by heavy metal stress in the *atpcs2-1* background to determine if expression levels are altered in comparison to wild type.

However one of the limitations of this study has been the characterization of only one loss-of-function allele. There are currently many different sources for obtaining a loss-of-function allele in a gene of interest such as:

http://signal.salk.edu/;

http://www.jic.bbsrc.ac.uk/science/cdg/exotic;

http://rarge.gsc.riken.go.jp/dsmutant/index.pl;

http://genetrap.cshl.org

It is therefore possible to obtain addition loss-of-function alleles in the *AtPCS2* gene for characterization. At the time of this publication, there were no known additional alleles of *AtPCS2* found from these sources.

In conclusion, the loss of the function of the *AtPCS2* gene did not have a detectable effect on cadmium sensitivity or metabolism in the *atpcs2-1* allele when

compared to wild type. The double mutant *cad1-3 atpcs2-1* also did not display a significant difference in comparison to *cad1-3* in all assays performed. Our conclusions support the current published results that *AtPCS2* does not play a significant role in heavy metal detoxification in comparison to *AtPCS1*.

III.

Long Distance Transport of Phytochelatins in *Arabidopsis*

1. Abstract

Phytochelatins (PCs) are peptides that function in heavy metal chelation and detoxification in plants and fungi. A recent study showed that phytochelatins have the ability to undergo long distance transport in a root to shoot direction in transgenic *Arabidopsis*. To determine whether long distance transport of phytochelatins can occur in the opposite direction, from shoots to roots, the wheat phytochelatin synthase (*TaPCS1*) gene was expressed under the control of a shoot specific promoter (*CAB2*) in an *Arabidopsis* PC-deficient mutant *cad1-3* (CAB2::*TaPCS1*/*cad1-3*). Analyses demonstrated that *TaPCS1* is expressed only in shoots and that CAB2::*TaPCS1*/*cad1-3* lines complement the cadmium and arsenic metal sensitivity of *cad1-3* shoots. CAB2::*TaPCS1*/*cad1-3* plants exhibited higher cadmium accumulation in roots and lower cadmium accumulation in shoots compared to wildtype. Fluorescence HPLC coupled to mass spectrometry analyses directly detected the phytochelatin PC2 in the roots of CAB2:*TaPCS1*/*cad1-3*, suggesting that PC2 is transported over long distances in the shoot to root direction. In addition, wildtype shoot tissues were grafted onto phytochelatin synthase *cad1-3 atpcs2-1* double loss-of-function mutant root tissues. An *Arabidopsis* grafting technique for mature plants was modified to obtain an 84% success rate, significantly greater than a previous rate of 11%. Fluorescence HPLC coupled to mass spectrometry showed the presence of the phytochelatins PC2, PC3, and PC4 in the root tissue of grafts between wild type shoots and *cad1-3 atpcs2-1* double mutant roots, demonstrating that phytochelatins are transported over long distances from shoots to roots in *Arabidopsis*.

2. Introduction

Heavy metals are defined as metals that have a density of greater or equal to 5.0 g cm^{-3} and include elements such as cadmium (Cd), lead (Pb), mercury (Hg), and copper (Cu). Some of these metals are essential at low concentrations, such as iron and copper, but at higher concentrations these metals can become toxic. High concentrations of heavy metals are serious environmental concerns, as the U.S. Environmental Protection Agency lists cadmium, mercury, lead, and the metalloid arsenic among the ten most hazardous contaminants at Superfund sites (http://www.atsdr.cdc.gov/clist.html).

A primary mechanism by which plants and fungi tolerate heavy metal toxicity is through the production of small thiolate peptides called phytochelatins (PCs) that bind a variety of metals (Kondo et al., 1984; Grill et al.1985). Phytochelatins, which have the chemical structure $(\gamma Glu\text{-}Cys)_n\text{-}Gly$ where n=2-11, are produced post-transcriptionally through the enzyme phytochelatin synthase (PCS) using glutathione as a substrate (Grill et al., 1989; Ha, et al., 1999; Clemens et al., 1999; Vatamanuik et al., 1999; Vatamanuik et al., 2000; Vatamanuik et al., 2004).

The *Arabidopsis* genome has two PCS genes: *AtPCS1* and *AtPCS2* (Clemens et al., 1999; Ha et al., 1999; Vatamanuik et al., 1999; Cazalé and Clemens, 2001; Lee and Kang, 2005). In the fungus *Schizosaccharomyces pombe*, complexes of heavy metals bound to PCs are transported across the tonoplast and sequestered in vacuoles by means of the ATP binding cassette transporter HMT1 (Ortiz et al., 1995). In plants PCs are also sequestered into vacuoles (Salt and Rauser, 1995). Therefore PCs were

predicted not to undergo long distance transport but to mainly aid in the sequestration of PC heavy metal complexes into vacuoles. Recently, phytochelatins were shown to undergo long distance transport in a root to shoot direction when a wheat phytochelatins synthase TaPCS1 (Clemens et al., 1999) was specifically targeted to *Arabidopsis* roots of the phytochelatin deficient *cad1-3* mutant using an *ADH1* promoter (Gong et al., 2003). Furthermore, root-targeted, non-native (*ADH1*) expression of TaPCS1 unexpectedly enhanced the accumulation of cadmium in the shoots and decreased cadmium accumulation in roots compared to the *cad1-3* controls (Gong et al., 2003).

To further analyze long distance PC transport abilities and the role of PCs in cadmium transport, here we address the question whether PCs have the ability to undergo long distance transport in the opposite direction from shoots to roots. The *cad1-3* mutant, which is a recessive loss of function mutant in the *Arabidopsis PCS1* gene and exhibits no detectable phytochelatins (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003), was transformed with a shoot specific promoter (*CAB2*) driving the expression of wheat TaPCS1 to determine whether shoot to root PC transfer is feasible. In addition, experiments were pursued to determine whether expression of *Arabidopsis PCS* genes under the spatial control of their native genomic promoters can mediate long distance shoot to root transport. For these analyses grafting techniques for mature *Arabidopsis* plants (Ayre and Turgeon, 2004) were modified for improved success rates and wildtype shoots were grafted onto *cad1-3 atpcs2-1* double loss-of-function mutants. Fluorescence HPLC and coupled parallel

mass spectrometry analyses of grafted plants demonstrate shoot to root transfer of phytochelatins.

3. Methods

DNA CONSTRUCTS AND PLANT TRANSFORMATION

The CAB2::*TaPCS1* plasmid was constructed by modifying the Adh::*TaPCS1*::c-myc/pBI121 binary expression vector (Gong et al., 2003) by digestion with the two restriction enzymes *Bam*HI and *Hind*III and subcloning in the CAB2 promoter to replace the Adh promoter. The DNA sequence for the promoter sequence of CAB2 was constructed by PCR recovery from a plasmid containing the CAB2 promoter. All PCR products were confirmed by sequencing (Retrogen, CA). The CAB2::*TaPCS1* construct was introduced into the PC deficient *Arabidopsis* mutant *cad1-3* by direct *Agrobacterium tumefaciens*-mediated transformation using the floral dip technique (Clough and Bent 1998).

PLANT MATERIAL, GROWTH CONDITIONS, AND METAL STRESS TREATMENTS

For growth in Petri dishes, *Arabidopsis* seedlings were grown on one-quarter-strength Murashige and Skoog (MS) basal medium (Sigma), 1 mM MES, 1% agar and the indicated concentrations of heavy metals (Lee et al., 2003). Seedlings used for shoot growth analyses were grown horizontally under 16/8-h day/night period and those used for root analyses were grown vertically under 24 hour light periods.

CAB2::*TaPCS1*/cad1-3 and grafted plants used for Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) and Fluorescence HPLC coupled to Mass Spectrometry analysis were grown in 80 mL of hydroponic medium as described (Arteca and Arteca, 2000) with minor modifications: Plants were germinated in Petri dishes and grown vertically for 4 days and moved to a horizontal position for an additional two days to bend the hypocotyl to aid in transplantation onto the hydroponic sponge. Plants were grown on sponges (Jaece Industries, Inc., North Tonawanda, New York) in Magenta boxes (Sigma) at 24°C under a 16/8-h day/night period and media were replaced every 3-4 days. After reaching bolting stage (approximately 4 weeks old), the media was replaced with 50 mL of the media to which 20 μM $CdCl_2$ was added for a period of 4 days for Cd^{2+}- accumulation analyses, whereas the media containing 20 μM $CdCl_2$ was added for a period of 3 days for induction of PCs.

For analysis of Cd^{2+}-dependent root growth, seeds were sterilized and plated on plates containing 25% MS medium, 1 mM MES, 1% Agar Type A (Sigma), cold treated at 4 °C for 48 hours, and grown vertically under 24 hour light growth room conditions for five days. Seedlings were then transferred to 25% MS, 1 mM MES, 1% Agar plates containing 20 μM $CdCl_2$ for an additional 72 hours of vertical growth.

GENERATION AND ISOLATION OF AN *ATPCS2* INSERTION MUTANT AND *CAD1-3 ATPCS2-1*

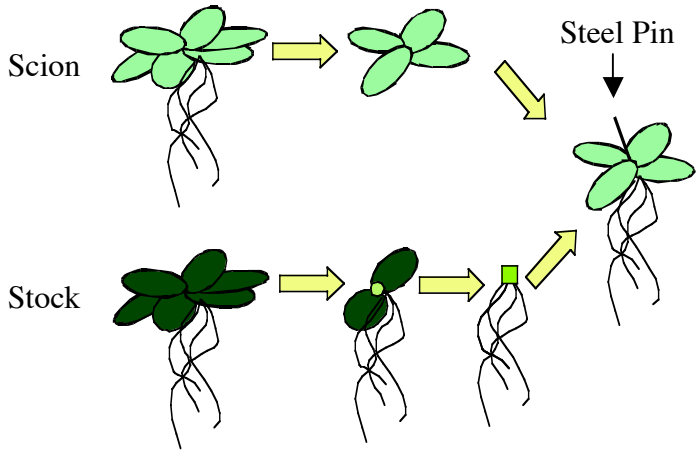
An *AtPCS2* T-DNA insertion mutant was isolated from the Wisconsin T-DNA collection by PCR screening (Krysan et al. 1999). A *cad1-3 atpcs2-1* double mutant

was generated by crossing *cad1-3* (Col background) with *atpcs2-1* (Ws background), and homozygous *cad1-3 atpcs2-1* lines were identified through PCR genotyping of the F2 population (data not shown). The isolation of the corresponding wild type for *cad1-3 atpcs2-1* was generated by crossing Ws to Columbia to generate an F2 population of Ws x Col.

GRAFTING OF MATURE *ARABIDOPSIS* PLANTS

Plants were grown under hydroponic conditions until the 6-8 rosette leaf stage (approximately 3 weeks) in 80 mL hydroponic medium (see above) in Magenta boxes under 16/8-h day/night period at 22^{o}C temperature conditions with the lids ajar to allow for air exchange. The component of the graft containing the root structure (stocks), were prepared for grafting using a transverse cut through the rosette stem above the first true rosette leaves using precision microscissors (Fine Science Tools, North Vancouver, Canada; Figure III-1A). The cotyledon and rosette leaves remaining on the stock were subsequently removed at the base of the petiole using microscissors. Scions, the portion of the graft not containing the root system, were prepared using a transverse cut through the rosette stem above the cotyledons (Figure III-1A). Scion and stock were attached using a steel pin (1 mm diameter, Fine Science Tools, North Vancouver, Canada) by impaling the center of the stem, and the pin was then inserted into the hydroponic sponge to secure the graft (Figure III-1A). The graft site, where the stock meets scion, was held approximately 0.25 cm above the hydroponic sponge by the steel pin to avoid adventitious root growth. Any grafts

A)



B)

Figure III-1. Grafting of Mature *Arabidopsis* plants. (A) Diagram of grafting technique. The upper portion illustrates preparation of portion of graft, labeled as "scion", not containing the root system, in which a transverse cut was made in the stem directly above the cotyledons (see Materials and Methods). The lower portion illustrates preparation of the stock, the portion of the graft containing the root system, in which a transverse cut was made in the stem directly above the first set of rosette leaves and the subsequent removal of the cotyledons and lowest rosette leaves. Scion and stock were secured together with a steel pin. (B) Two grafted *Arabidopsis* plants are shown 10 days post-grafting after a 72 hour exposure to 20 μM $CdCl_2$. Depicted is a graft between wild type (Ws x Col-O F2) shoots and *cad1-3 atpcs2-1* roots. All floral organ growth occurred post-grafting and illustrates new organ development in grafts.

observed to have adventitious root growth were immediately discarded. The lids of the Magenta boxes were then closed to insure high humidity for 7 days. Grafts that produced new leaves and floral organs post-grafting were scored as successful grafts (Figure III-1B).

Several of the grafts that were scored as successful, by initiating new organ development post-grafting, displayed purple leaves, which were likely due to anthocyanin production and interpreted as indicators of stress (Figure III-1B). Wild type shoots of such stressed individuals also showed transfer of PCs to the *cad1-3 atpcs2-1* double mutant roots. Grafting experiments to analyze long distance shoot to root PC transfer were initially developed using plants 10 days post grafting, which included a 3 day 20 μM cadmium treatment. However, 7 days post grafting, including a 3 day 20 μM cadmium treatment, proved to be a better time period for PC analyses and these conditions were used for the illustrated PC analyses.

NORTHERN BLOTTING AND RT-PCR

Total RNA was extracted from shoots and roots using the TRIzol reagent (Invitrogen). RNA gel blotting, probe labeling, and hydridizations were accomplished using standard protocols recommended by manufacturers. Northern blots were probed with the *Actin 7* (At5g09810) gene as a loading control and with *TaPCS1*. First-strand cDNA was synthesized from DNAseI-digested total RNA using Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and PCR performed on a

PE GeneAmp 9700 (PE Applied Biosystems) with 50 PCR cycles using Biolase *Taq*DNA polymerase (DocFrugal, San Diego, CA).

INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY ANALYSES

Cadmium treated plants grown under hydroponic conditions were first washed and then separated into root and shoot tissues. Shoot tissues were separated immediately below the cotyledons and above the hydroponic sponge. Root tissues were separated below the hydroponic sponge, approximately 3 cm below cotyledons. Shoots were rinsed three times in deionized water. For ICP-OES analyses of root tissue, roots were rinsed in deionized water, washed in 100 mL of 100 mM $CaCl_2$ on an orbital shaker (Bellco Glass, Inc., NJ) for 5 minutes at approximately 135 rotations/min (speed setting #4), and then washed in 100 mL of deionized water on the orbital shaker (speed setting #4) for an additional three minutes. Both shoot and root tissues were dried at 60^{o}C overnight. The dry weight was recorded, and then the tissues were digested in 70% trace metal grade nitric acid (Fisher Scientific) overnight. Samples were then boiled for 30 minutes to ensure complete digestion and diluted to a final concentration of 5% nitric acid with deionized water.

PC ANALYSES

CAB2::*TaPCS1*/*cad1-3* plants were grown under hydroponic conditions until they bolted (approximately 4 weeks) and then incubated in hydroponic media

containing 20 μM $CdCl_2$ for 72 hours. 20 μM $CdCl_2$ was added to successfully grafted plants seven days post grafting. Plants were rinsed in three times in deionized water, root and shoot tissue separated, and lyophilized for 24 hours. Shoot and root tissues were separated as described in Materials and Methods see: Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analyses. Phytochelatins were and derivatized with mono-bromobimane (mBBr) as described (Clemens et al., 1999; Sneller et al., 2000). Synthesized standards were used for the identification of PCs (γ-EC)$_2$G (PC_2), (γ-EC)$_3$G (PC_3), (γ-EC)$_4$G (PC_4). The fluorescence HPLC column (Ultrasphere C18 ion-pair HPLC column; Beckmann Catalog #235335) was directly coupled to the Mass Spectrometer (LC/MS) by splitting the 1 mL/min flow in which 900 μL/min of the flow went to the fluorescence detector (FL3000 Fluorometer; Spectrasystem) and HPLC (Surveyor LCQ Advantage; ThermoFinnigan; Waltham, MA), and 100 uL/min was electrosprayed in parallel directly into the mass spectrometer (Surveyor LCQ Advantage; ThermoFinnigan; Waltham, MA). PC standards were run after every fifth sample to monitor the migration and changes in retention time of PC peaks due to normal alterations in the properties of the HPLC column over time.

PHLOEM SAP EXTRACTION

Four to five mature rosette leaves were detached at their petiole bases. The petioles were recut under 20 mM EDTA (pH 7.5). All leaves collected from one plant were placed in a 1.5 ml microcentrifuge tube with their petioles immersed in 1.0 to

1.25 ml of 15 mM EDTA (pH 7.5) (Berthomieu et al., 2003). Tubes were placed in airtight transparent plastic containers in which the atmosphere is water-saturated to prevent uptake of the EDTA solution by the leaves for 4 hrs in an illuminated growth room to dissolve the phloem sap in the EDTA solution (Corbesier et al., 1998; Corbesier et al., 2003; Berthomieu, P. et al., 2003). Then the EDTA solution containing phloem sap was lyophilized and reconstituted in 30 µL of water and labeled with monobromobimane for PC analysis.

XYLEM SAP EXTRACTION

Plants were grown hydroponically and treated with 20 µM $CdCl_2$ at bolting stage for 72 hours. Plants on hydroponic floaters were placed in a container filled with deionized water. All rosette leaves are removed with scissors, and the inflorescence stem was cut with a very sharp razor blade (Gaymard et al., 1998; Shi et al., 2002). The tray and plants were covered with a transparent plastic dome. Xylem sap drops were collected using a micropipette as the droplets accumulate at the cutting surface of the inflorescence stem (Gaymard et al., 1998; Shi et al., 2002). Xylem sap was used directly for PC analysis.

4. Results

SHOOT SPECIFIC EXPRESSION OF WHEAT *TAPCS1* CDNA IN *CAD1-3*

The *Arabidopsis cad1-3* mutant, containing a defective *AtPCS1* gene was selected as the background to target wheat *TaPCS1* expression because the mutant

lacks detectable PCs (Cobbett et al., 1998; Howden et al., 1995; Gong et al., 2003). The wheat gene *TaPCS1* was used to avoid co-silencing, as it only has a 49.2% nucleotide identity to *AtPCS1*. A 199 bp region of the *CAB2* promoter was linked to the wheat *TaPCS1* cDNA to drive expression of *TaPCS1*, and *cad1-3* plants were transformed with this construct. Sixteen independent homozygous lines of CAB2::*TaPCS1*/*cad1-3* were isolated, and three independent lines used for further analysis were selected based on showing the highest levels of *TaPCS1* mRNA transcript as determined by Quantitative RT-PCR of the sixteen isolated homozygous lines isolated (data not shown).

Northern blot analyses showed that expression of *TaPCS1* mRNA was specific to shoot tissue in all three independent lines (Figure III-2A). Shoot specific expression of wheat *TaPCS1* was confirmed in the RT-PCR experiments (Figure III-2B). No *TaPCS1* mRNA was detected in roots tissue even after 50 cycles of RTPCR (Figure III-2B). In contrast, the positive control 35s:*TaPCS1*/*cad1-3* line showed strong expression of *TaPCS1* mRNA in both shoot and root tissue (Figure III-2A).

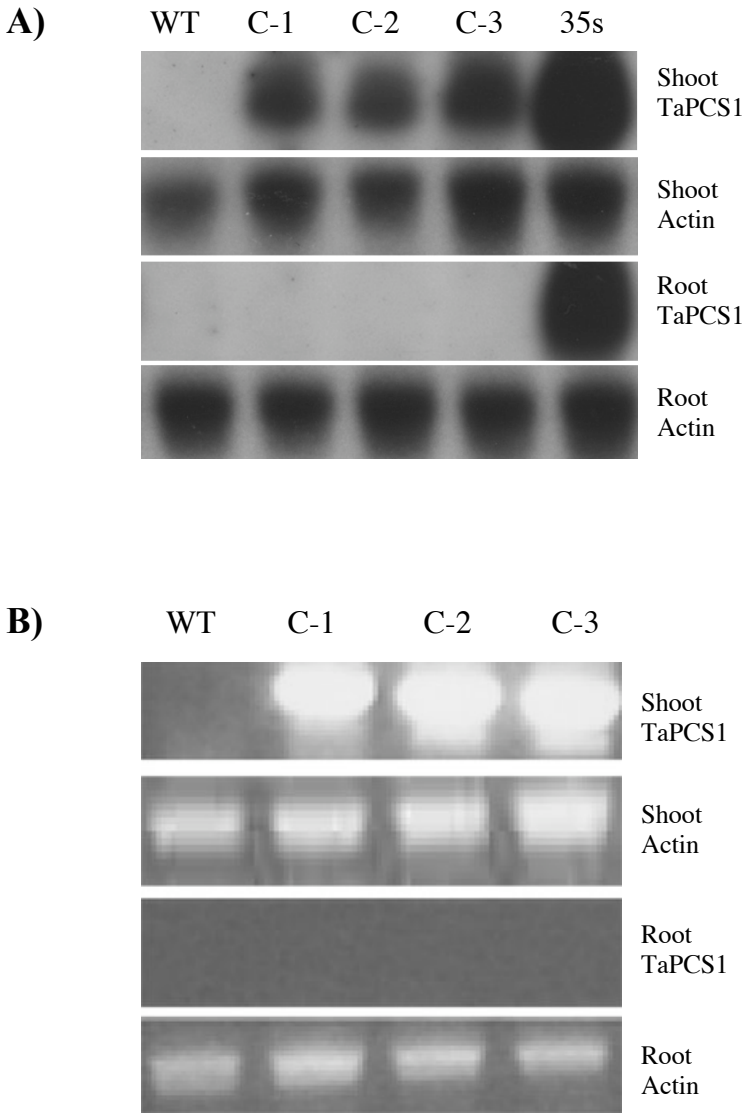


Figure III-2. Expression of *TaPCS1* mRNA is targeted to the shoots of CAB2::*TaPCS1*/*cad1-3* plants. (A) Northern blots probing *TaPCS1* expression in WT (Col-O), three independent lines of CAB2::*TaPCS1*/*cad1-3* (C-1, C-2, C-3), and 35s::*TaPCS1*/*cad1-3* (35s). Actin was used as a loading control (n=2). (B) 50 cycles of RT-PCR show lack of any *TaPCS1* expression in roots and strong *TaPCS1* expression in shoots. RT-PCR was performed with *TaPCS1*::c-myc fusion-specific primers. Actin7 primers used as a loading control (n=2).

TRANSGENIC EXPRESSION OF CDNA COMPLEMENTS HEAVY METAL SENSITIVITY OF *CAD1-3* IN SHOOT TISSUE BUT NOT IN ROOT TISSUE

cad1-3 was unable to grow on plates containing 40 μM $CdCl_2$ or 80 μM KH_2As0_4 (Figure III-3C and 3D; n=60 seedlings) which correlates with the lack of detectable phytochelatins in *cad1-3*. Experiments showed that the shoot sensitivity of *cad1-3* can be complemented by both the CAB2::*TaPCS1* or 35S::*TaPCS1* constructs, as both of these lines produced green cotyledons as did wild type seedlings when grown on cadmium or arsenate (Figure III-3C and 3D).

Root growth of the *cad1-3* mutant was also highly sensitive to cadmium in comparison to wildtype seedlings (Figure III-4). The three CAB2::*TaPCS1*/*cad1-3* lines (C-1, C-2, and C-3) were not able to complement the root sensitive phenotype of *cad1-3*, and showed enhanced growth inhibition by Cd^{2+} compared to wild type (Figure III-4). However, lines C-2 and C-3 showed significantly longer root growth in comparison to *cad1-3* (C-2 $P = 3.7 \times 10^{-4}$; C-3 $P = 2.2 \times 10^{-6}$).

PHYTOCHELATINS ARE TRANSPORTED IN A SHOOT TO ROOT DIRECTION

To determine whether PCs can undergo long distance transport in the shoot to root direction, PC levels in shoot and root tissues were analyzed in the three independent CAB2::*TaPCS1*/*cad1-3* lines as well as positive and negative controls. Fluorescence HPLC analyses of monobromobimane labeled phytochelatins from these tissues was performed to analyze the levels of PCs (Figure III-5). PC2, PC3, and PC4

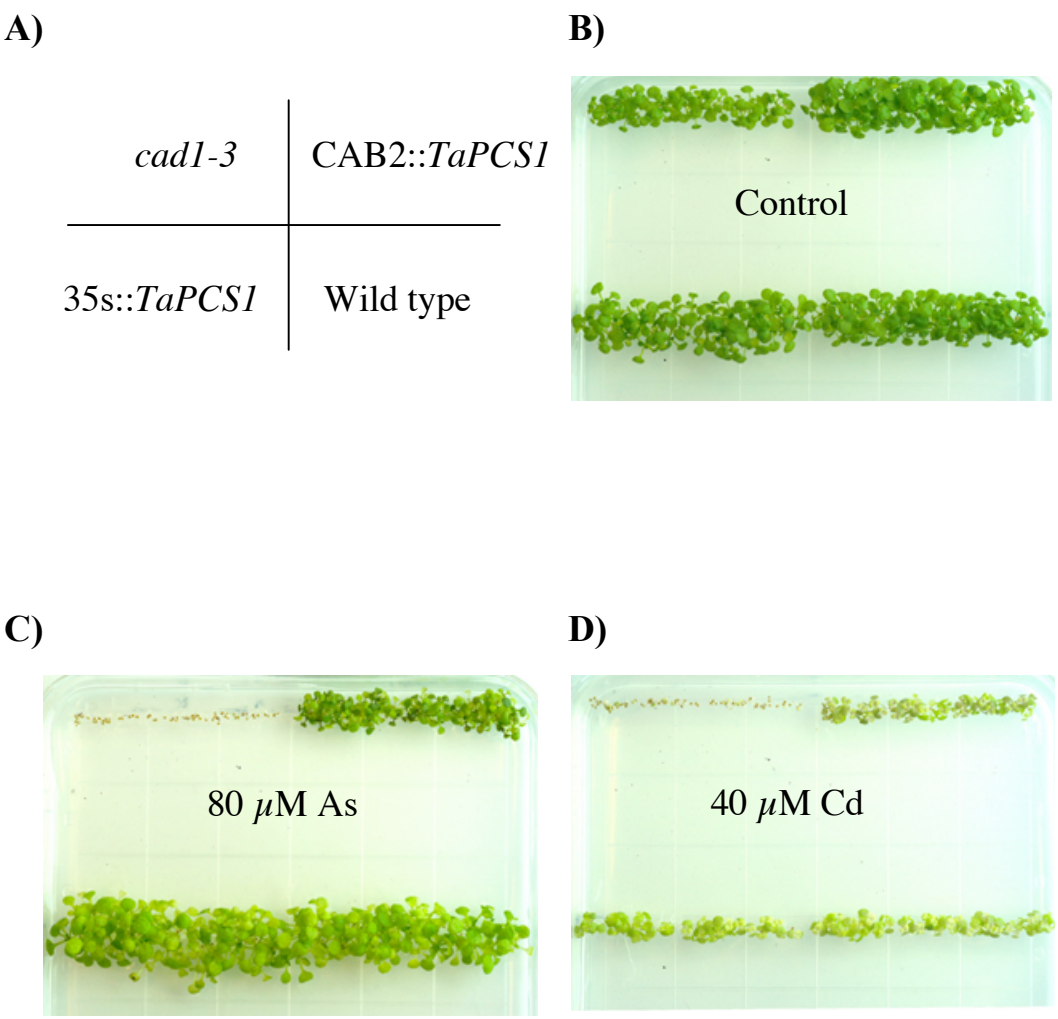


Figure III-3. CAB2::*TaPCS1* expression complements the cadmium and arsenic sensitivity of *cad1-3* in shoots. For each panel, seeds of four different lines were germinated as diagramed in (A): *Upper left, cad1-3 (atpcs1)*; *Upper right,* CAB2::*TaPCS1* (CAB2::*TaPCS1*/*cad1-3*); *Lower left*, 35s::*TaPCS1* (35s::*TaPCS1*/*cad1-3*), *Lower Right*, Wild type (Col-O ecotype). All seeds were germinated and were grown on one-quarter-strength Murashige and Skoog medium for 14 days on either (B) medium containing no heavy metal as a "Control", or (C) 80 μM As, (D) or 40 μM Cd.

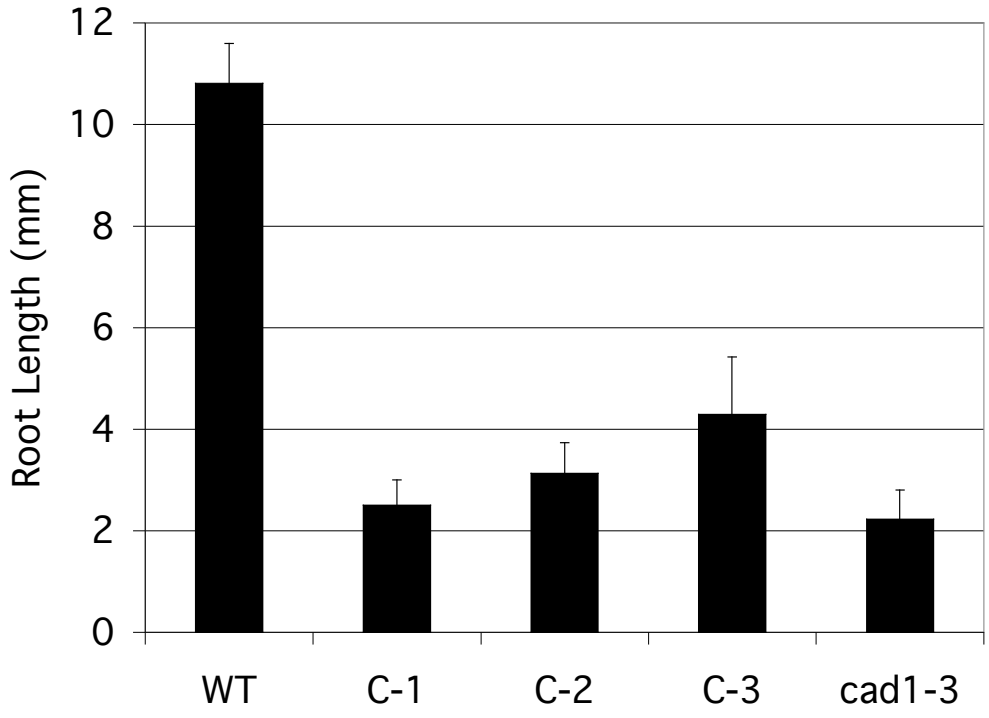


Figure III-4. CAB2::*TaPCS1* expression in shoots of *cad1-3* does not complement the cadmium sensitivity of the root growth of *cad1-3*. Wild type, three CAB2::*TaPCS1*/*cad1-3* lines (C-1, C-2, C-3), and *cad1-3* seedlings were germinated on one-quarter strength Murashige and Skoog with no added cadmium for 5 days and then transferred to plates containing 20 μM $CdCl_2$ for 3 days. Root length presented is a measure of new root growth after the transfer of seedlings to plates containing 20 μM $CdCl_2$. Data show mean values ± SEM; n= 60 plants per plant line.

peaks were identified using synthesized PC standards and are shown in panels G and H of Figure III-5. The HPLC flow was split to the fluorescence detector and to a mass spectrometer to simultaneously identify the molecular weights of fluorescence HPLC peaks (Figure III-6). As previously reported (Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003), *cad1-3* plants did not display any detectable phytochelatins even when the fluorescence detector gain was amplified (Figure III-5E and F; n=14 of 14 plants). PCs were clearly detected in positive control wildtype shoot and root tissues of 4 week old plants exposed to cadmium (Figure III-5A and B; n=15 of 16 plants). In all three CAB2::*TaPCS1*/cad1-3 lines PC2, PC3, and PC4 were detected in shoot tissue (Figure III-5C; n=38 of 40 plants). Interestingly the phytochelatin PC2 was clearly detected in the root tissue of all three CAB2::*TaPCS1*/*cad1-3* lines (Figure III-5D; n=38 of 40 plants). As *TaPCS1* mRNA was exclusively expressed in shoot tissues of the CAB2::*TaPCS1*/*cad1-3* lines (Figure III-2), the presence of PC2 in the roots indicated that PC2 underwent long distance shoot to root transport in the transgenic CAB2::*TaPCS1*/*cad1-3*.

To identify and verify the presence of phytochelatins in the root tissue of CAB2::*TaPCS1*/*cad1-3* plants, peaks eluted from the HPLC were analyzed by mass spectrometry. The predicted and observed mass for PC2 standard labeled with two monobromobimane molecules at the +1 ion state was 920 m/z (Figure III-6A; n=30). Peaks of identical mass were observed in both wild type and CAB2::*TaPCS1*/*cad1-3* samples (Figure III-6B C; n=15 of 16 plants for wild type; n=38 of 40 plants for CAB2::*TaPCS1*/*cad1-3*). The larger background present in the CAB2::*TaPCS1*/cad1-

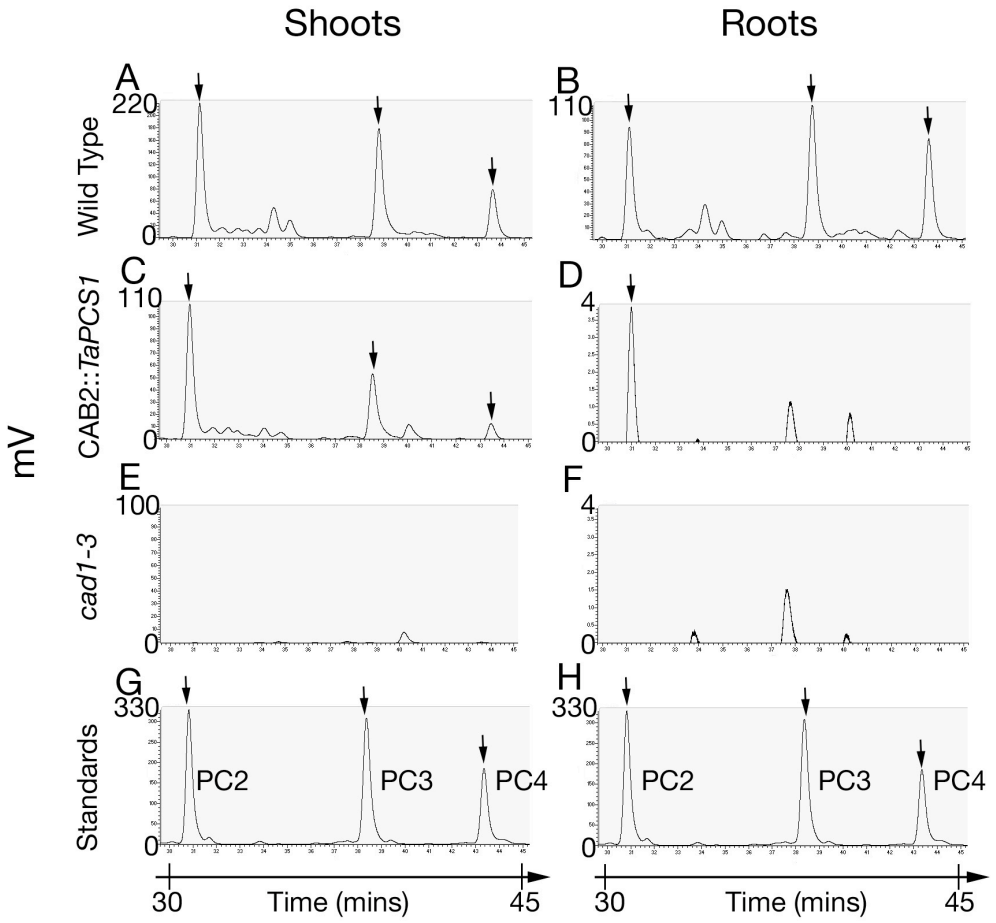


Figure III-5. Phytochelatins detected in shoot tissue and PC2 in root tissues of CAB2::*TaPCS1*/*cad1-3* plants. Four week old plants grown in hydroponic conditions were exposed to 20 μM $CdCl_2$ and PCs in shoot and root tissue extracts were labeled with monobromobimane [shoot tissue extracts left column (A, C, E); root tissue extracts right column (B, D, F)]. PC2, PC3, and PC4, indicated by arrows, were detected by fluorescence HPLC and compared to synthesized standards (G, H). Note that the positions of arrows indicating PC peaks were calibrated using PC standard control experiments after every fifth sample. The slight shifts in PC peak retention times from experiment to experiment are due to the normal changes in the properties of the HPLC column over time. PC standards in G and H are identical and shown twice for visual analysis of all traces. (E, F) *cad1-3* served as negative controls with a 2-fold magnified Y-axis and (A, B) WT (Col-O) served as positive control. n = 13 to 14 plants were analyzed for each of the three CAB2::*TaPCS1*/*cad1-3* lines.

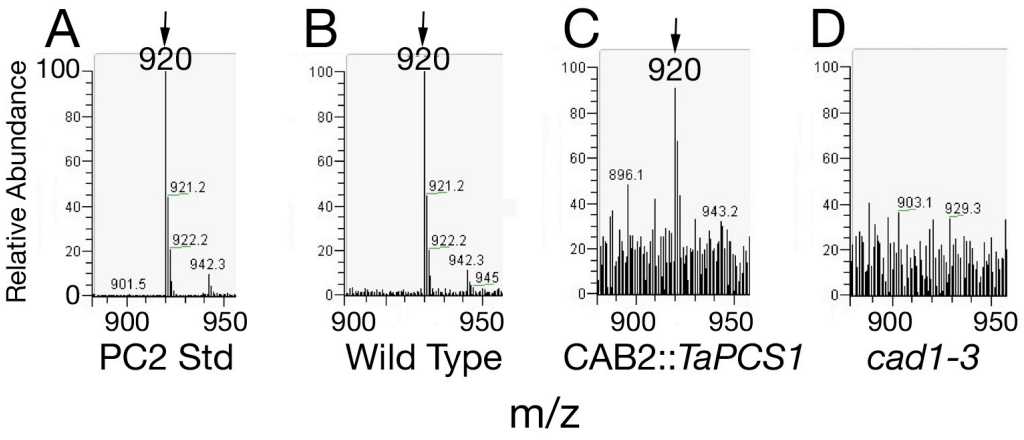


Figure III-6. Mass spectrometry run concurrently with Fluorescence HPLC confirms HPLC peaks as phytochelatins in root tissues. A to D show data from root samples. PC2 conjugated with two monobromobimane molecules showed a value of 920 m/z (z = ion charge) as indicated by arrows. (A) Synthesized PC2 standard, (B) Root sample from wild type (Col-O), (C) Root sample from CAB2::*TaPCS1*/*cad1-3*, (D) Root sample *cad1-3*.

3 suggested a lower level of PC2 in the sample. In negative control experiments, no 920 m/z peak was found in the *cad1-3* extracts (Figure III-6D; n=14 plants), supporting the hypothesis that PC2 is transported from shoots to roots in CAB2::*TaPCS1*/*cad1-3* plants.

CADMIUM ACCUMULATES IN ROOT TISSUE OF CAB2::*TAPCS1*/*CAD1-3*

Cadmium levels in root and shoot tissues of 4 weeks old plants exposed to 20 μM $CdCl_2$ for 4 days were analyzed by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). *cad1-3* control plants showed Cd^{2+} over-accumulation in roots and reduced Cd^{2+} accumulation in shoots compared to wild type plants (Figure III-7). These data are consistent with previous findings under different conditions that showed a contribution of *AtPCS1* (*CAD1*) to Cd^{2+} transfer from roots to shoots (Gong et al., 2003). CAB2::*TaPCS1*/*cad1-3* shoots showed slightly higher accumulation of cadmium in shoot tissues in comparison to *cad1-3* (Figure III-7, grey bars; C-1 $P = 5.1 \times 10^{-4}$; C-2 $P = 9.1 \times 10^{-5}$; C-3 $P = 7.9 \times 10^{-3}$). However, the overall effect of cadmium distribution from shoot specific expression of *TaPCS1* in *cad1-3* was relatively minor compared to *cad1-3*, but showed large differences when compared to wild type plants.

In CAB2::*TaPCS1*/*cad1-3* roots, higher concentrations of Cd^{2+} were observed in CAB2::*TaPCS1*/*cad1-3* roots in comparison to wild type roots (Figure III-7; n=9 plants per line; C-1 $P = 1.1 \times 10^{-4}$; C-2 $P = 2.1 \times 10^{-4}$; C=3 $P = 4.1 \times 10^{-3}$). Lower concentrations of PCs were detected in root and shoot tissue of the CAB2::*TaPCS1*/*cad1-3* (Figure III-5C and D) in comparison to wild type. The lower

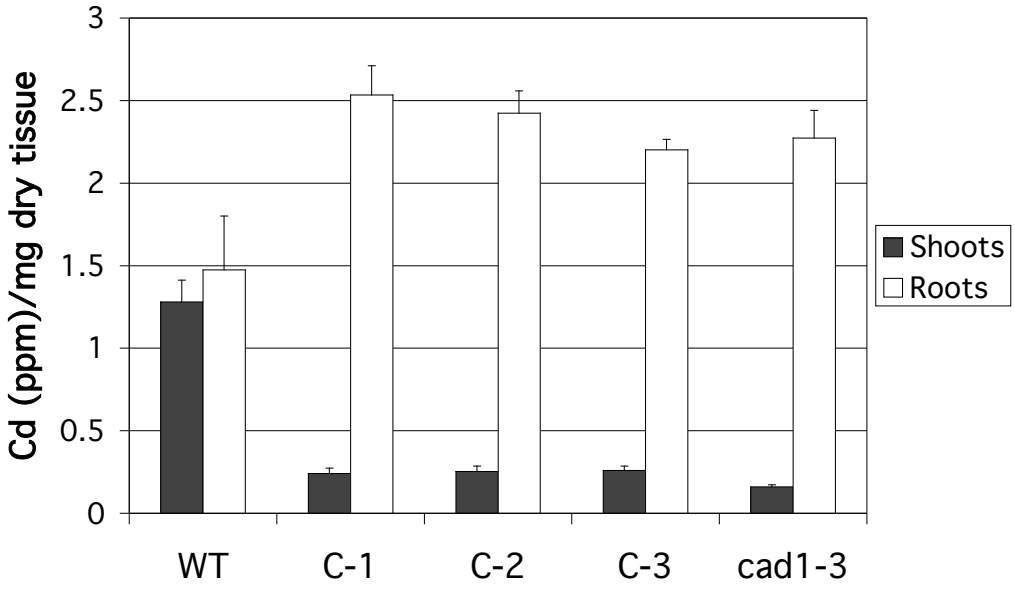


Figure III-7. Cadmium over-accumulation in roots and under-accumulation in shoots of CAB2::*TaPCS1*/*cad1-3* and *cad1-3* plants. Wild Type (Col-O), three independent lines of CAB2::*TaPCS1*/*cad1-3* (C-1, C-2, C-3), and *cad1-3* were grown under hydroponic conditions and exposed to 20 µM $CdCl_2$ for 4 days. Cd^{2+} accumulation in shoot and root tissues was determined by ICP-OES. Data show mean values ± SEM, n=9 plants per line.

PC concentrations found in the roots of CAB2::*TaPCS1*/*cad1-3* compared to wild type plants (Figure III-5B and D) correlates with the dramatically lower concentration of cadmium being translocated into shoot tissues of CAB2::*TaPCS1*/*cad1-3* in comparison to wild type (Figure III-7). Thus CAB2::*TaPCS1*/*cad1-3* and *cad1-3* plants showed similar cadmium accumulation patterns in both shoots and roots (Figure III-7), which may be attributable to the absence of PCs in roots during the initial Cd^{2+} exposure period of CAB2::*TaPCS1*/*cad1-3* roots (see Discussion).

GRAFTS DEMONSTRATE SHOOT TO ROOT PHYTOCHELATIN TRANSFER

In the present study grafting experiments were pursued in mature plants to analyze long distance transport of PCs in whole plants. Grafting techniques were originally developed for *Arabidopsis* seedlings (Turnbull, et al., 2002). A previously developed successful grafting procedure for mature *Arabidopsis* plants showed a success rate of approximately 11% (n= 13 of 120 plants; Ayre and Turgeon, 2004). Modifications to the published grafting techniques in mature plants in the present study led to an initial 84% (n=22 of 25 plants) success rate after 10 days of growth. The high grafting success rate can be attributed to several modifications, including the growth of plants on hydroponic media in Magenta boxes rather than soil, which allowed a more sterile and humid environment (See Materials and Methods). In addition, this method does not require the bending of a steel pin for graft stabilization (Ayre and Turgeon, 2004), because the dense hydroponic sponge in which the plants are grown has the ability to securely hold the pin and grafted tissues together in

comparison to soil grown plants (Figure III-1A). The use of a transverse cut with precision microscissors (Turnbull et al., 2002) also simplified the grafting technique and increased the success rate. The development of grafting in mature plants required that grafts show the ability to survive 10 days post grafting and to initiate new organ development (Ayre and Turgeon, 2004). Figure III-1B depicts grafts between wild type shoots and *cad1-3 atpcs2-1* roots 10 days post grafting, showing new organ development.

The CAB2::*TaPCS1*/*cad1-3* results presented above provide evidence that non-native *CAB2* promoter driven expression of the wheat *TaPCS1* cDNA enables long distance shoot to root transport of phytochelatins. However the question whether *Arabidopsis* phytochelatin synthases expressed under their native promoters enable shoot to root PC transport remains unknown. To directly analyze this question and to further investigate the deduced shoot to root transport, grafting experiments were performed with a double mutant in both *Arabidopsis AtPCS* genes. A T-DNA insertion mutant in the *AtPCS2* gene was isolated from the Wisconsin T-DNA population (Krysan et al., 1999) and PCR screening of an F2 population led to the isolation of a homozygous T-DNA insertion in the *AtPCS2* gene (*atpcs2-1*). *atpcs2-1* contains a T-DNA insertion in the sixth intron of the *AtPCS2* gene. RT-PCR analysis was performed which demonstrated the absence of a full length mRNA transcript in *atpcs2-1*.

Experiments consisting of shoot growth and root growth response analyses to cadmium, cadmium accumulation, and PC quantification suggested that *atpcs2-1* had

no easily discernable phenotype in comparison to its wildtype ecotype (WS) under the imposed conditions. These data are consistent with studies indicating a putative minor or unknown function of *AtPCS2* (Cazalé and Clemens 2001; Lee and Kang, 2005). As expected, the *cad1-3 atpcs2-1* double mutant plants showed similar phenotypes to *cad1-3* plants and produced no detectable phytochelatins when analyzed with fluorescent HPLC coupled to mass spectrometry (data not shown; n=13 of 13 plants). To directly test the ability of natively expressed *AtPCS* genes to mediate long distance phytochelatin transport in the shoot to root direction, 3 week old shoot (scion) tissues from wild type plants (Ws x Col F2 individuals) were grafted to 3 week old *cad1-3 atpcs2-1* double mutant root (stock) tissues. Positive control grafts between wild type shoots and wildtype roots and negative control grafts between *cad1-3 atpcs2-1* shoots and *cad1-3 atpcs2-1* roots were also performed.

Extracts from grafted plants containing wildtype shoot and *cad1-3 atpcs2-1* root tissue were labeled with monobromobimane and were analyzed by fluorescence HPLC coupled to mass spectrometry. Interestingly, PC2, PC3, and PC4 were detected in the roots of *cad1-3 atpcs2-1* grafted to wild type shoots indicating transport of PCs in a shoot to root direction (Figure III-8D; n=12 of 20 root samples). Wild type shoots (Ws x Col F2 individuals) were grafted onto wild type roots (Ws x Col F2 individuals) to serve as a positive control and showed PCs in roots and shoots (Figure III-8A and B; n=7 of 12 for PCs in shoots; n=10 of 12 plants for PCs in roots). *cad1-3 atpcs2-1* shoots were grafted onto *cad1-3 atpcs2-1* roots to serve as a negative control and showed no PCs (Figure III-8E and F; n=9 of 9 plants).

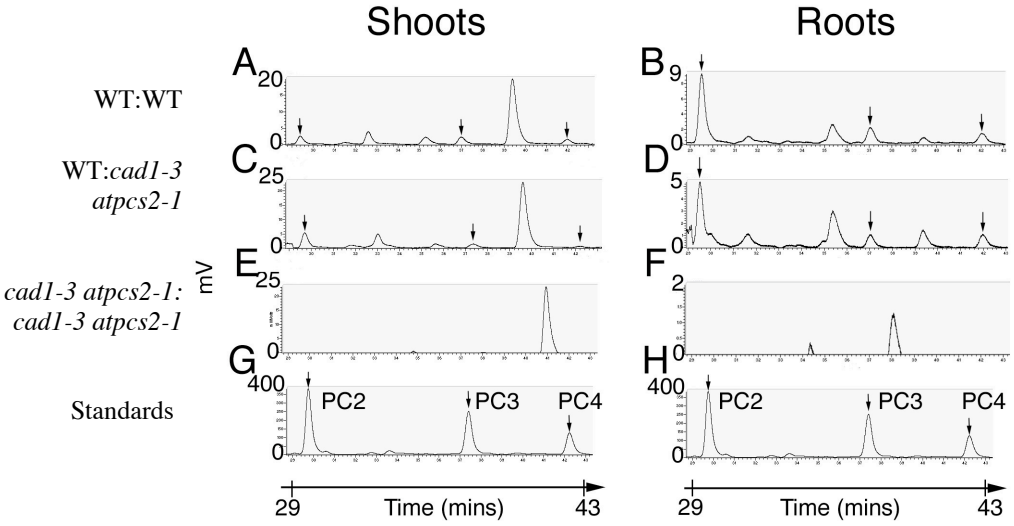


Figure III-8. Phytochelatins are transported from shoots to roots in grafts between wild type shoots and *cad1-3 atpcs2-1* double mutant roots. Plant were grown under hydroponic conditions and tissues extracted for PC analyses prepared 7 days post grafting which included a 3 day exposure to 20 μM $CdCl_2$ [shoot tissue extracts left column (A, C, E) ; root tissue extracts right column (B, D, F)]. PCs were labeled with monobromobimane and detected by fluorescence HPLC. (A, B) Grafts between wild type (Ws x Col-O F2) shoots and roots (n= 7). (C, D) Grafts between Wild Type (Ws x Col-O F2 individuals) shoots and *cad1-3 atpcs2-1* roots (n= 12). Note that the Y-axes are not identical and amplified in D and F. (E, F) Grafts between *cad1-3 atpcs2-1* shoots and roots (n=9). (G, H) Synthesized PC standards. Note that the positions of arrows indicating PC peaks were calibrated using PC standard control experiments after every fifth sample. The slight shifts in PC peak retention times from experiment to experiment are due to the normal changes in the properties of the HPLC column over time. G and H are identical traces and shown twice to facilitate visualization of above fluorescence HPLC traces. Y-axes represent milliVolts (mV) and x-axes represent retention time (minutes).

We identified and confirmed the fluorescent HPLC peaks using directly coupled mass spectrometry (LC/MS). The mass spectrometer analyses of the PC2 standard labeled with two monobromobimane molecules showed the predicted mass of the +1 ion at 920 m/z (Figure III-9G); PC3 standard labeled with three monobromobimane molecules showed the predicted mass of the +2 ion at 672 m/z (Figure III-9H); and the PC4 standard labeled with four monobromobimane molecules showed the predicted mass of the +2 ion at 883 m/z (Figure III-9I). In plants with wildtype shoots grafted to *cad1-3 atpcs2-1* roots, directly coupled mass spectrometry showed peaks and masses corresponding to PC2, PC3, and PC4 in both shoots (data not shown) and roots (Figure III-9A to C; n=12 of 20 root samples). Positive control grafts between wild type (Ws x Col) shoots and wild type roots (Ws x Col) showed peaks with identical masses corresponding to these PCs in coupled mass spectrometry (data not shown). Whereas negative control grafts between *cad1-3 atpcs2-1* shoots and *cad1-3 atpcs2-1* roots showed no detectable PCs (Figure III-9G to I; n=9 of 9 plants). The presence of PCs in *cad1-3 atpcs2-1* root tissues that had been grafted to wild type shoots (Figure III-9A to C), which were clearly absent in the *cad1-3 atpcs2-1* mutant (Figure III-9D to E), demonstrate that PCs are natively transferred in a shoot to root direction in *Arabidopsis*.

5. Discussion

The role of phytochelatins in mediating heavy metal detoxification in plants and fungi is well established (Kondo et al., 1984; Grill et al., 1985; Grill, 1987; Ortiz

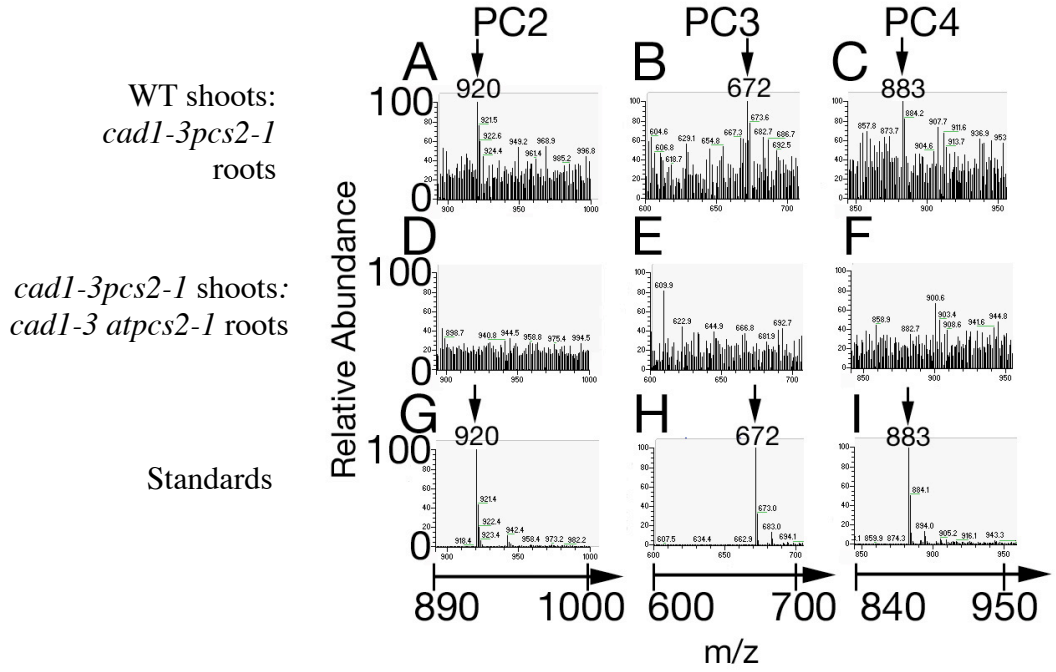


Figure III-9. Mass spectrometry ran concurrently with Fluorescence HPLC confirms PCs in root samples. A to F show data from root samples. (A, B, and C) Raw mass spectrometry data from root samples from plants with wild type shoots grafted to *cad1-3 atpcs2-1* roots; (D, E, and F) Data from root samples from plants with *cad1-3 atpcs2-1* shoots grafted to *cad1-3 atpcs2-1* roots; (G, H, and I) PC standards. PC2 at the +1 ion state was 920 m/z (z = ion charge), PC3 at the +2 ion state was 672 m/z, and PC4 at the +2 ion state was 883 m/z as indicated by arrows. Y-axis represents relative abundance percentage and x-axis represents mass per ion charge (m/z) as labeled.

et al., 1992; Ortiz et al., 1995; Howden et al., 1995; Ha et al., 1999; Cobbett, 2000). A recent study has shown that PCs have the ability to travel in a root to shoot direction when wheat TaPCS1 was expressed under the control of the ADH promoter in *Arabidopsis* roots of phytochelatin-deficient plants (Gong et al., 2003). *cad1*-3 controls show enhanced Cd^{2+} accumulation in roots which was reduced by TaPCS1 expression in *cad1-3* roots (Gong et al., 2003). The present study focused on two main questions: (1) Can phytochelatins be transferred in the opposite direction from shoots to roots in *Arabidopsis*, and (2) Grafting experiments addressed the question: Is native promoter expression of *Arabidopsis PCS* genes in shoots sufficient for shoot to root transport of phytochelatins to occur, or does the native shoot to root response differ from the non-native CAB2 promoter-directed *TaPCS1* expression. We have found that the phytochelatin PC2 [$(\gamma\text{-EC})_2$G] can travel in a shoot to root direction using non-native CAB2::*TaPCS1* expression (Figures III-4 and III-5). In addition, root tissues of CAB2::*TaPCS1*/*cad1-3* plants over-accumulate cadmium at levels similar to *cad1-3* compared to wild type plants (Figure III-6). To analyze PC shoot to root transport, we adapted and further developed a grafting method in mature plants resulting in a substantial increase in the percentage of viable grafted plants. To further unequivocally test whether phytochelatins can be transported from shoots to roots, we pursued grafting experiments. Grafting experiments demonstrated shoot to root transfer of the phytochelatins $(\gamma\text{-EC})_2$G (PC_2), $(\gamma\text{-EC})_3$G (PC_3), and $(\gamma\text{-EC})_4$G (PC_4) with natively expressed *AtPCS* genes in shoots.

ATPCS1 AIDS IN CADMIUM TRANSLOCATION

Previous research and the present study showed that the phytochelatin-deficient mutant *cad1-3* (*AtPCS1*) (Howden et al., 1995; Ha et al., 1999) shows increased accumulation of Cd^{2+} in roots compared to wild type (Figure 6; Gong et al., 2003). In the present study CAB2::*TaPCS1*/*cad1-3* lines also showed Cd^{2+} over-accumulation in roots compared to wild type controls, even though CAB2::*TaPCS1*/*cad1-3* plants contained PC2, PC3, and PC4 in shoots (Figure III-5C) and transferred PC2 to roots (Figure III-5D).

Heavy metals are required to activate PC synthesis in plants (Grill 1987; Maitani et al., 1996; Vatamaniuk et al., 2000). The over-accumulation of Cd^{2+} in roots of CAB2::*TaPCS1*/*cad1-3* plants (Figure III-7) may be due to lack of phytochelatin production directly in roots during initial cadmium exposure before the transport of PC2 from the shoot tissues of CAB2::*TaPCS1*/*cad1-3* occurred.

Roots are the first tissues to experience cadmium toxicity. In roots, cadmium has been observed to damage nucleoli, alter the synthesis of RNA, inhibit ribonuclease activity (Shah and Dubey, 1995) and reduce absorption of nitrate by inhibiting the nitrate reductase activity in shoots (Hernandez et al., 1997). CAB2::*TaPCS1*/*cad1-3* seedlings grown in the presence of cadmium display less root growth and are therefore more sensitive to cadmium in comparison to wild type seedlings (Figure III-4). However the roots of two of the three independent lines of CAB2:*TaPCS1/cad1-3* (C-2 and C-3) were slightly longer in comparison to *cad1-3* (Figure III-4). This may be due to the presence of low levels of transported PC2 to root tissues from shoot

tissues (Figures III-4 and III-5). Thus cadmium-damaged *cad1-3* root tissues have a reduced ability to effectively transport cadmium to shoots.

In contrast to CAB2::*TaPCS1*/*cad1-3* and *cad1-3* plants, root specific *TaPCS1* expression, or 35s::*TaPCS1*/*cad1-3*, and wild type plants express an active version of PCS in root tissues at the time of initial cadmium exposure, and concomitantly show reduced Cd^{2+} accumulation in roots and enhanced Cd^{2+} accumulation in shoots (Figure 6; Gong et al., 2003). In addition, the low levels of PC2 observed in the root tissues of CAB2::*TaPCS1*/*cad1-3* (Figure III-5) are likely not sufficient to effectively affect the root to shoot balance of Cd^{2+} transport to shoot tissues. The large differences in Cd^{2+} accumulation in roots and shoots between *cad1-3* (*atpcs1*) and wild type plants provide direct genetic evidence for a role of *AtPCS1* in Cd^{2+} distribution in *Arabidopsis* (Figure III-7).

PCS OVEREXPRESSION IN WILD TYPE BACKGROUNDS

Recent reports have analyzed a different important question than the present study, namely whether overexpression of *PCS* cDNAs in wild type backgrounds (rather than in *cad1-3* mutants) can enhance metal resistance (Gisbert et al., 2003; Lee et al., 2003b; Sauge-Merle et al., 2003; Li et al., 2004; Pomponi et al., 2005). These studies address the questions of (1) whether wild type phytochelatin synthase activities are already saturated in the wild type background of the analyzed plant species and (2) whether single *PCS* gene overexpression can further enhance traits including heavy metal resistance. These studies have shown interesting and differential results

depending on the analyzed plant species and applied toxic metals. For example, overexpression of *AtPCS1* in *Arabidopsis* wild type plants caused sensitivity to Cd^{2+} in two studies (Lee et al., 2003b; Li et al., 2004). But interestingly the same plants in one of these studies showed a dramatically enhanced resistance to arsenic (Li et al., 2004). In another study, overexpression of the *Arabidopsis AtPCS1* cDNA in wild type tobacco and glutathione feeding showed different results, enhancing both Cd^{2+} resistance and Cd^{2+} accumulation in shoots and roots (Pomponi et al., 2005). In addition, transgenic expression of the wheat *TaPCS1* gene in *Nicotiana glauca* (shrub tobacco) increased lead uptake and accumulation and cadmium tolerance (Gisbert et al., 2003).

Thus different plant species show different responses to single gene *PCS* overexpression and it appears that multigene approaches may be needed to dramatically enhance Cd^{2+} accumulation in transgenic wild type plants, as intermediates, such as cysteine and glutathione, can limit the production of phytochelatins (Clemens et al., 2002). In support of a multigenic systems approach to enhancing heavy metal metabolism, in *Escherichia coli* co-overexpression of three proteins in the PC biosynthesis pathway: PCS, serine acetyltransferase, and gamma-glutamylcysteine synthase resulted in the over-accumulation of phytochelatins and cadmium in bacterial cells and showed that single gene overexpression in the PC biosynthetic pathway had limited effects (Wawrzynska et al., 2005).

The present study and a previous study (Gong et al., 2003) did not analyze TaPCS1 overexpression in wild type backgrounds, but focused on long distance

phytochelatin transport by characterizing effects of transgenic *TaPCS1* expression and grafting relative to PC deficient mutant lines. This approach allows direct genetic analyses of long distance phytochelatin transport and Cd^{2+} distribution analyses relative to phytochelatin deficient mutant controls.

SHOOT TO ROOT TRANSPORT OF PHYTOCHELATINS AND POSSIBLE TRANSPORT MECHANISMS

In order to directly analyze whether phytochelatins have the ability to undergo long distance shoot to root transport, we generated and analyzed double loss-of-function mutant plants in the two *Arabidopsis* PCS genes, *AtPCS1* (*CAD1*) (Ha et al., 1999; Vatamanuik et al., 1999; Clemens et al., 1999) and *AtPCS2* (Cazalé and Clemens, 2001; Lee and Kang, 2005). As expected the *cad1-3 atpcs2-1* double mutant plants showed no detectable phytochelatins in 13 plant samples analyzed (data not shown) similar to the *cad1-3* single gene mutant (Figures III-5E and F; III-6B; Howden et al., 1995; Cobbett et al., 1998; Gong et al., 2003). Grafting of *cad1-3 atpcs2-1* double mutant roots to wild type shoots clearly showed transfer of the phytochelatins PC2, PC3, PC4 from shoot to roots (Figures III-8C and D; III-9D and F).

The mechanisms by which PCs undergo long distance transport in plants remain unknown. Previous research in *Brassica juncea* showed that cadmium was mainly found in xylem sap extracts as complexes with nitrogen containing compounds that are not reminiscent of phytochelatins (Salt et al., 1995). The present study shows

that PCs undergo long distance transport in a shoot to root direction, which cannot be mediated by the xylem and implicates phloem transport as a long distance transport pathway. Phloem and xylem sap were extracted from wild type plants (Ws ecotype) treated with cadmium to induce the production of phytochelatins and analyzed for the presence of phytochelatins (See Materials and Methods). No phytochelatins were detected in either phloem or xylem sap, which suggests either the sap extraction method was not optimal for PC analysis or PCs are not found in either xylem or phloem sap. Phloem transport of phytochelatins would likely require several types of phytochelatin transporters for phloem loading and unloading which remain unknown. Phytochelatins are small peptides, and recent completion of the *Arabidopsis* genome has revealed a number of potential peptide transporter families for phytochelatins. Peptide transporters have been placed into two groups based on their energy source: (1) the oligopeptide transporter (OPT) and peptide transporters (PTR) families which use proton-motive force, and (2) ATP-binding cassette (ABC-type) transporters which use ATP hydrolysis as an energy source.

A member of a OPT proton-motive force transporter family was shown to translocate tetra- and pentapeptide substrates when expressed in yeast (Koh et al., 2002). Expression of AtOPT4 in *Schizosaccharomyces pombe* mediated the uptake of Lys-Leu-Gly-[^{3}H]Leu (Koh et al., 2002). In addition, the *Saccharomyces cerevisiae* oligopeptide transporter, ScOPT1, was recently shown to display a higher affinity for PC2 in comparison to reduced or oxidized glutathione and oligopeptides including the terapeptide GGFL (Osawa et al., 2005).

Members of a different proton-motive force transporter family, PTR, which is also known as the proton oligopeptide transporter (POT) family, have shown the ability to transport small peptides. For example, AtPTR1 recognizes a broad spectrum of di- and tripeptides, is localized to the plasma membrane, and is expressed in vascular tissues throughout the *Arabidopsis* plants suggesting a role in long-distance peptide transport (Dietrich et al., 2004).

The ABC-type transporter superfamily is represented by a large gene family in *Arabidopsis* with approximately 130 members (Sanchez-Fernandez et al., 2001). ABC-type transporters transport substrates ranging from small ions to large macromolecules (Sanchez-Fernandez, et al., 2001). In *S. pombe*, the heavy metal tolerance factor 1 (*SpHMT-1*) gene encodes an ABC-type transporter and has been shown to mediate uptake of PC- Cd^{2+} complexes into *S. pombe* vacuoles (Ortiz et al., 1995). Recently a HMT1 homolog in *Caenorhabditis elegans* was identified (CeHMT-1), and was shown to be required for cadmium tolerance (Vatamaniuk et al., 2005). *Arabidopsis* lacks a direct HMT1 homologue. To date the identification of an *Arabidopsis* gene(s) encoding the vacuolar transporter for PCs has not been reported. A MgATP-energized transport pathway for PCs and PC- Cd^{2+} complexes, analogous to ABC-type transporter, has been characterized in vacuolar membrane vesicles isolated from oat roots (Salt and Rauser, 1995). Recently the *Arabidopsis* ABC-type transporter AtPDR12 was shown to contribute to lead resistance by serving to exclude lead and/or lead containing compounds (Lee et al., 2005).

Phytochelatins may also serve as signaling molecules to communicate heavy metal content between different tissue types. PCs transported from shoots to roots (Figures III-5, III-6, III-8 and III-9), for example, might serve as a signal for roots to down-regulate nutrient transporters in roots to prevent further uptake of heavy metals. Cadmium competes with the physiological transport of nutrients such as calcium, iron, magnesium, manganese, copper and zinc, as Cd^{2+} is transported by transmembrane nutrient transporters in plants (Clarkson and Lüttge 1989; Riveta et al., 1997; Clemens et al., 1998; Grotz et al., 1998; Curie et al., 2000; Picard et al., 2000; Thomine et al., 2000; Clemens et al., 2002; Thomine et al., 2003; Papoyan and Kochian 2004).

In conclusion, long distance shoot to root transport of phytochelatin peptides was found in the present study in non-native promoter driven CAB2::*TaPCS1*/*cad1-3* and native promoter driven grafts between wild type shoots and *cad1-3 atpcs2-1* roots. Shoot specific targeting of wheat *TaPCS1* in *cad1-3* restores cadmium and arsenic tolerance of leaves and does not significantly alter the cadmium over-accumulation phenotype in the roots of *cad1-3* mutant plants. In addition, improved grafting techniques were developed for mature *Arabidopsis* plants to successfully analyze long distance transport of phytochelatins. Further characterization of shoot to root long distance PC transport mechanisms and transport regulatory pathways for phytochelatins will elucidate the functions and pathways of PCs in heavy metal processing in plant biology.

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The text of Chapter Three, in part, is a reprint of the materials that has been submitted for publication. The dissertation author was the primary researcher and author and co-authors, Julian I. Schroeder and Elizabeth A. Komives, listed in this publication directed and supervised the research which forms the basis of this chapter.

IV.

Conclusions

The work presented in this thesis was undertaken with the goal to further understanding the molecular mechanisms plants employ to deal with cadmium stress from their environment and provide further insight into the role that phytochelatins play in heavy metal detoxification and translocation.

Chapter I describes the isolation and characterization of cadmium tolerant *Arabidopsis* mutants through the development of a novel post-germination screen. Cadmium was chosen for a variety of reasons. First, it is one of the ten most toxic compounds found at contaminated sites in the United States (Johnson and Derosa 1995). Second, in comparison to other heavy metals cadmium is one of the most widely studied in plants yet there are many aspects of cadmium toxicity in plants that are not known (Sanita di Toippi and Gabbrielli, 1998). Third, there have been no reports of genetic screens performed to isolate genes that specifically confer tolerance to cadmium. The post-germination screen was utilized to screen through populations mutagenized by the chemical mutagen EMS and populations containing activation tags. EMS mutagenized mutant Cd11 showed greater shoot and root development in the presence of cadmium in comparison to wild type. In addition, Cd11 accumulated higher levels of cadmium in both root and shoot tissue in comparison to wild type. Mutants screened from activation tagged lines also showed greater shoot and root development in the presence of cadmium. Genes identified through this cadmium tolerance screen could potentially be used to enhance the phytoremediation potential of plants through genetic modification.

Chapter II summarizes the isolation and characterization of a loss-of-function allele in the *AtPCS2* gene (*atpcs2-1*). *atpcs2-1* did not display a significant difference in comparison to wild type in regards to shoot and root development in the presence of cadmium, cadmium accumulation and distribution pattern, or phytochelatins levels. These results suggest that *AtPCS2* does not play a major role in cadmium detoxification. In addition to *atpcs2-1*, a *AtPCS* double mutant, *cad1-3 atpcs2-1*, was generated and characterized. *cad1-3 atpcs2-1* did not display a significant difference when compared to the *AtPCS1* single mutant, *cad1-3* as both did not produce detectable phytochelatins, exhibited similar shoot and root growth phenotypes in the presence of cadmium, and accumulated and distributed similar levels of cadmium. These results also confirm the hypothesis that *AtPCS2* does not play a major role in cadmium detoxification in comparison to *AtPCS1*.

Chapter III provides further insight into the role phytochelatins play in heavy metal toxicity. Phytochelatins were believed to be sequestered inside the plant cell, however a recent publication has shown that phytochelatins have the ability to travel from the root to shoot direction (Gong et al., 2003). Both shoot specific targeting of the wheat *TaPCS1* gene (CAB2::TaPCS1/*cad1-3)* and grafting experiments between wild type shoots and *cad1-3 atpcs2-1* roots have demonstrated that phytochelatins also travel in the opposite direction, namely from shoots to roots. In addition, shoot specific targeting of *TaPCS1* in the *cad1-3* background showed over-accumulation of cadmium in roots similar to the cadmium accumulation seen in *cad1-3*. CAB2::TaPCS1 also showed resistance in shoot growth, but sensitivity in root growth

in comparison to *cad1-3*. The ability of phytochelatins to undergo long distance transport poses a possible phytochelatin dependent mechanism for heavy metal long distance transport as phytochelatins have been shown to bind to heavy metals (Grill 1985). The mechanisms plants use to transport heavy metals are not well known in plants. As the success of phytoremediation requires targeting pollutants to ariel portions of the plant, a long term application of these findings would be to genetically modify plants to enhance the targeting of heavy metals to the shoots.

The molecular mechanisms that plants employ to deal with heavy metal stress is not well characterized in plants. Phytoremediation is a promising alternative to traditional remediation techniques, and a further research into the molecular mechanisms could potentially aid in genetically enhance plants to remove pollutants from the environment.

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