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Isolation and Characterization of Cadmium Hypertolerant Mutants of *Arabidopsis Thaliana*

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ISOLATION AND CHARACTERIZATION OF CADMIUM HYPERTOLERANT MUTANTS OF *ARABIDOPSIS THALIANA*

by

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Santiago Xavier Navarro

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Cadmium is an environmental pollutant which accumulates by deposition from anthropogenic activities. Plants readily take-up cadmium from soil and is concentrated in shoot tissues. Plant and animal systems are affected detrimentally by the exposure to cadmium and are known to produce metal binding peptides as part of detoxification mechanisms. In an attempt to find alternative methods of soil clean-up, efforts are being undertaken to isolate heavy metal accumulating plants. Alternatively, to reduce the accumulation of cadmium in food, plants that exclude heavy metals are also being sought. The objective of this project was to isolate mutants which can be used to elucidate these metal detoxification processes.

Two cadmium hypertolerant mutants of *Arabidopsis thaliana*, *cdht1* and *cdht4*, have been isolated using a Vertical Mesh Transfer system (Murphy & Taiz, 1995). Exposure to cadmium inhibits the growth of roots of wild type plants. This phenotype was used to isolate mutants which are able to grow their roots >2mm upon exposure to 200 μM cadmium. Genetic analysis indicates that the *cdht1* mutant has a dominant phenotype when compared to the wild type. On the other hand, *cdht4* mutant plants exhibit a recessive phenotype when compared to the wild type. Both of the phenotypes from isolated mutant lines segregate as a single Mendelian locus. Assays of cadmium accumulation using ICP-MS indicate that *cdht1* and *cdht4* mutants are cadmium-excluders in relation to wild type.

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CHAPTER I

LITERATURE REVIEW

Cadmium in the Environment

Heavy metals, such as cadmium, lead, and mercury, can be environmental pollutants that act as toxicants in plant and animal systems. Cadmium (Cd), a naturally occurring element found in the earth's crust and most waters, results from events such as emissions into the air from volcanic activity and cycling release by vegetation (Robards & Worsfold, 1991). Increasing heavy metal concentrations in soils have been observed in areas where industrial processes occur, leading to contaminated sites which leave both land unusable for agriculture and causes general toxicity to many biological systems (EPA, 1987; Wozny et al., 1990). Reported levels of Cd in soil vary greatly between continents and are usually not homogeneous in distribution. In the U.S., representative reports on the concentration of Cd are available from the U.S. Department of Energy (DOE). U.S. DOE facilities are numerous throughout the United States, half of which have been reported to be contaminated with toxic elements such as Cd. Cd levels at U.S. DOE facilities range between 0.1 and 345 $\mu g\ g^{-1}$ of dry weight soil between uncontaminated and contaminated sites respectively (Cornish et al., 1995).

Input and Bioavailability

Major sources of Cd input into the environment include air emissions by anthropogenic activity, such as the burning of fossil fuels, cigarette smoke, mining,

smelters, and electroplating (EPA, 1987; Prasad, 1995; Seaward & Richardson, 1990). Cd emissions accumulate in soil by the process of deposition. Soil contamination with Cd occurs also by the amendment of agricultural soils with municipal sludge and phosphate fertilizers (Mortvedt, 1995; Prasad, 1995; Seaward & Richardson, 1990). Once in the environment, heavy metals show long persistence (as they are not biodegradable) and become available for uptake by many plant species. Plants possess a wide range of tolerance mechanisms which are used to survive a variety of environmental conditions. Unfortunately, one of these mechanisms is the ability to remove toxic heavy metals from soil and store these metals inside plant cells (Rauser, 1995). Bioavailability of the metal for bioaccumulation and plant uptake is dependent on the chemical form of the metal, soil pH, soil organic matter content (primarily humic and phobic acids), salinity, cation exchange capacity of the soil, and other physiochemical properties (Prasad, 1995; Robards & Worsfold, 1991).

Cadmium Toxicity

Health Effects on Humans

In humans, Cd is not known to have any beneficial effects but rather has adverse health effects. Prolonged exposure to low Cd levels may result in adverse effects to the kidneys, bones, lungs, testes, the immune system, the nervous system, and the cardiovascular system (Nwosu et al., 1995; Robards & Worsfold, 1991). Cd may enter the body by inhalation and skin absorption, but most intake occurs by the ingestion of contaminated plant material (Gartrell et al., 1986). The toxicity of this metal depends on the chemical and physical forms of the element and the level of exposure. The soluble forms of cadmium, such as $CdCl_2$, are better absorbed and

therefore more toxic than the insoluble forms, such as CdS (Rusch et al., 1986). Organisms exposed to heavy metals produce sulfur rich metal binding peptides, grouped in the metallothionein super family, which help reduce the amount of free Cd in the system and aid in the process of detoxification (Grill et al., 1987). Once Cd is chelated in the body, it tends to be bioconcentrated and has the potential of affecting human health (i.e. itai-itai disease caused by ingestion of Cd-contaminated rice) (Groten & Vanbladeren, 1994).

Phytotoxicity

Inhibition of enzyme activity, such as in Rubisco (Ribulose 1,5 bisphosphate carboxylase/oxygenase), carbonic anhydrase, and phosphoribulokinase (Van Assche & Clijsters, 1990; Prasad, 1995), is one of the primary physiological effects of Cd in plants. Enzymes are inhibited by the interaction of available SH groups with the Cd^{2+} ion, leading to a change in the conformation of the enzyme. Secondary effects of Cd toxicity include a decrease in photosynthetic rates, total biomass, root growth, and germination (Van Assche & Clijsters, 1990; Rascio et al., 1993; Prasad, 1995). Cd generally reduces water stress tolerance, cell wall elasticity, transpiration, and stomatal resistance (Prasad, 1995). Exposure to Cd has also been reported to cause leakage of potassium from fine root cells, reduction in cellular levels of magnesium and calcium, and is able to inhibit the uptake of nutrient ions such as iron (Prasad, 1995; Eide et al., 1996; Gussarson et al., 1996; Yang et al., 1996). Experimental data have shown that treatment of plants with Cd causes the induction of heat-shock proteins (HSP) (in soybean (Edelman et al., 1988), Alfalfa cell cultures (Gyorgyey et al., 1991), and *Arabidopsis* (Takahashi & Komeda, 1992)), peroxidases and related stress response enzymes, such as glucose-

6-dehydrogenase, probably as a result of oxidative stress and depletion of glutathione (De Vos & Schat, 1981; Van Assche & Clijsters, 1990; Chen & Kao, 1995). Toxicity has also been associated with the appearance of a red pigment in the above ground shoots (Cumming & Tomsett, 1992).

Cadmium Tolerance in Plants

Mechanisms of Tolerance

Adaptability and tolerance mechanisms in plants are ubiquitous, although levels of tolerance and mechanisms of tolerance vary widely among species (Prasad, 1995). Possible mechanisms of tolerance include two major categories, accumulation or exclusion of the toxicant. Exclusion mechanisms include reduced metal uptake due to alterations in membrane composition (i.e. negatively charged membrane components and available SH content), ion transport (i.e. differential uptake by ATPase), release of chelating material, and the efflux of the metal out of the root (Baker & Walker, 1990; Verkleij & Schat, 1990; Cumming & Tomsett, 1992; Prasad, 1995; de la Fuente et al., 1997). Accumulation mechanisms include the production metal chelating substances, compartmentalization, and alterations in cell wall composition (Baker & Walker, 1990; Verkleij & Schat, 1990; Wozny et al., 1990; Cumming & Tomsett, 1992; Prasad, 1995).

Plant Influx, Transport, and Accumulation

Plants readily take up Cd from the soil, presumably through symplastic transport across the root (Figure 1). Cd is translocated to the shoots following the formation of a metal-protein complex with chelating peptides. The Cd is then bioconcentrated in vacuoles (Prasad, 1995). Cd is thought to interact with

negatively charged cell membrane components by non-specific binding. Then, Cd enters the cells by active transport mechanisms of ion uptake (Prasad, 1995). In some cases, Cd may be preferentially accumulated in the roots instead of the shoots (Cataldo et al., 1983). This suggests that uptake and transport of heavy metals may be species specific and that each process is independently regulated.

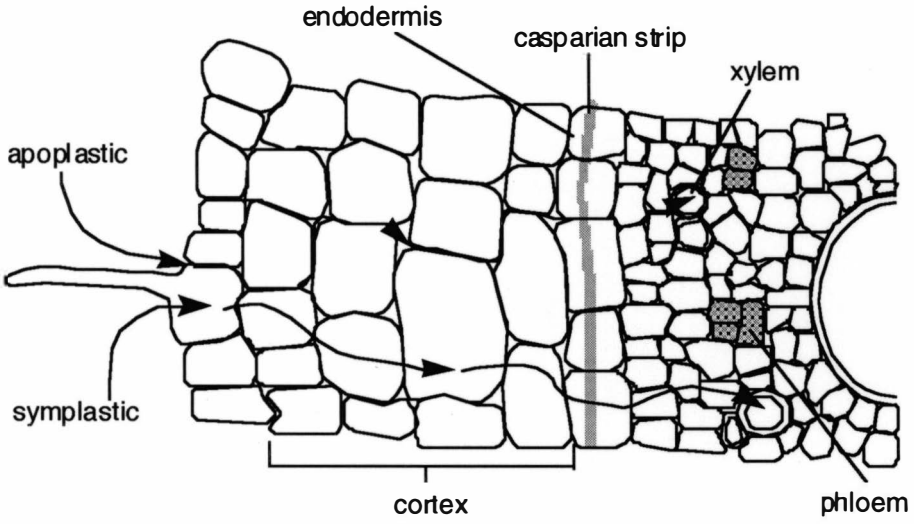
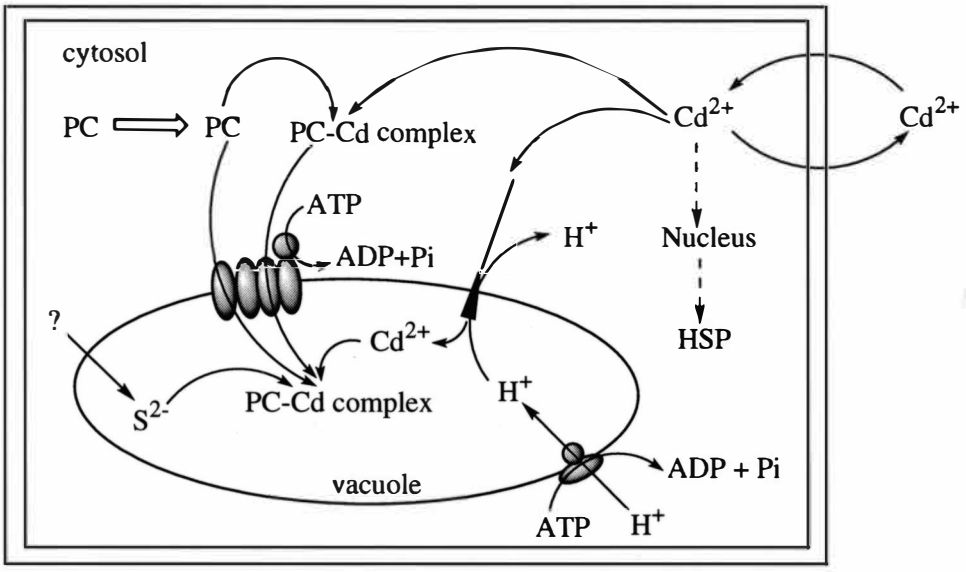


Figure 1. Symplastic and Apoplastic Pathways of Ion Absorption Into the Root. Sketch adapted from Salisburry and Ross (1991).

In vascular plants, translocation of Cd to the shoots is regulated by vascular tissues as movement occurs through vascular bundles and appears to be transpiration driven (Salt et al., 1995). The Cd-peptide complexes are then stored in vacuoles as a detoxification mechanism (Vögeli-Lange & Wagner, 1990; Salt & Wagner, 1993; Salt & Rauser, 1995). Studies on oat roots showed that low molecular weight peptides (γ-Glu-Cys) and Cd-peptide complexes are taken from the cytosol into the vacuole through an ATP-binding cassette type transporter (Salt & Rauser, 1995)(Figure 2). Additionally, entry of Cd^{2+} ions into vacuoles of oat roots has been shown to occur through a H^+ ATPase generated vacuolar proton

gradient and a Cd^{2+}/H^{+} antiporter system (Salt & Wagner, 1993). Low molecular weight peptides combine with Cd^{2+} and sulfide in the vacuole to form high molecular weight complexes (Rauser, 1995) (figure 2). Thus, accumulation of Cd in plants depends on the uptake by the root system, water relations, and availability of Cd binding peptides.



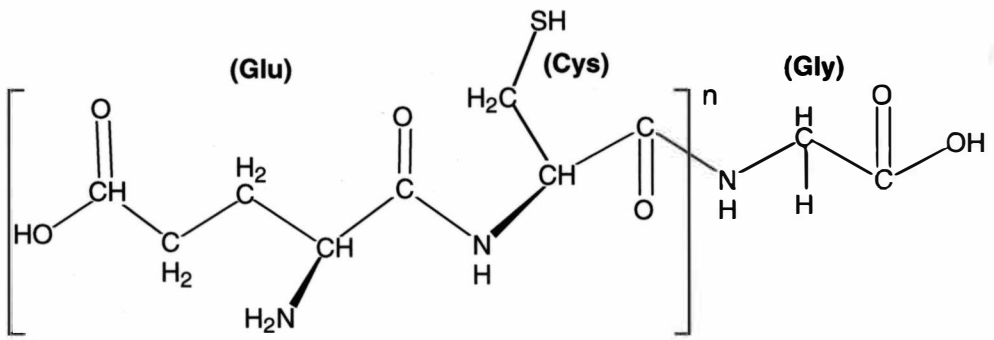
Legend. PC= Phytochelatins, HSP= Heat shock proteins

Figure 2. Model of Cellular Detoxification Responses to Cd. Redrawn from models described by Wozny et al. (1990) and Rauser (1995).

Plant Chelating Peptides

Plants generally respond to heavy metal contaminants by the production of metal binding peptides (Grill et al., 1987). Plant metal binding peptides are type III metallothioneins, functional homologues of the mammalian metallothionein with the primary structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n=2-12 (Figure 3) (Rauser,

1990; Robinson, 1990; Rauser, 1995). Some plants also produce other minor metal binding peptides, members of the type III metallothioneins superfamily, which have the primary structure $(\gamma\text{-Glu-Cys})_n$, $(\gamma\text{-Glu-Cys})_n$-Ser, $(\gamma\text{-Glu-Cys})_n$-Glu, or $(\gamma\text{-Glu-Cys})_n$-Ala (Rauser, 1995). The major form of these peptides generally referred to as phytochelatins (PCs), although some synonyms include cadystins, Cd-thioneins, Cd binding peptides, and class III metallothioneins (Wozny et al., 1990). Unlike mammalian metallothioneins, type III metallothioneins are not directly encoded by structural genes since they contain γ-carboxyamide type bonds which are not known to be synthesized by ribosomes (Rauser, 1990).



Legend. Glu= Glutamate, Cys= Cysteine, Gly= Glycine

Figure 3. General Chemical Structure of Phytochelatins. Repeating units of Glu-Cys are depicted here by the brackets, where n represents the number of repeats.

Phytochelatins are capable of binding heavy metals *in vitro* and are rapidly induced *in vivo* by the presence of these metals, supporting the idea that PCs play an important role in the detoxification mechanism of heavy metals (Rauser, 1990; Howden & Cobbett, 1992). The inducible production of metal binding peptides seems to result from the activation of phytochelatin synthase, the enzyme that catalyzes the

transpeptidation of glutathione with glutamylcysteinyl moieties of glutathione for the production of phytochelatins (Figure 4) (Grill et al., 1989; Grill et al., 1991). The activity of phytochelatin synthase appears to be self-regulated by the presence of activating metal ions, which become unavailable as the metal ions are chelated (Rauser, 1995). Phytochelatin synthase is constitutively expressed and is not induced noticeably upon exposure to Cd (Rauser, 1995). In the absence of toxic levels of metals, phytochelatins are also accumulated in metal resistant and metal sensitive cell cultures (Wozny et al., 1990). These results suggest that phytochelatins may play a role in the regulation of trace metal homeostasis (Wozny et al., 1990; Rauser, 1995).

The utilization of L-buthionine sulfoximine (BSO) as an inhibitor of the biosynthesis of glutathione (Figure 4 and 5), has been shown to increase the sensitivity of plant cells to heavy metals (Howden et al., 1995a), an effect that has been reversed upon the addition of glutathione (Mendum et al., 1990). BSO acts as an irreversible inhibitor of the enzyme γ–glutamylcysteine synthetase, presumably by causing allosteric changes due to its binding to phosphate groups. This, along with the disappearance of glutathione concomitant to the accumulation of phytochelatins, supports the idea that glutathione is a precursor of phytochelatins (Rauser, 1995).

Cd complexes with phytochelatins and accumulates in the vacuoles of plant cells, although the mechanism of transport to the shoots remains unclear (Salt & Wagner, 1993). This evidence suggests that PCs play an important role in the heavy metal detoxification mechanisms in plants.

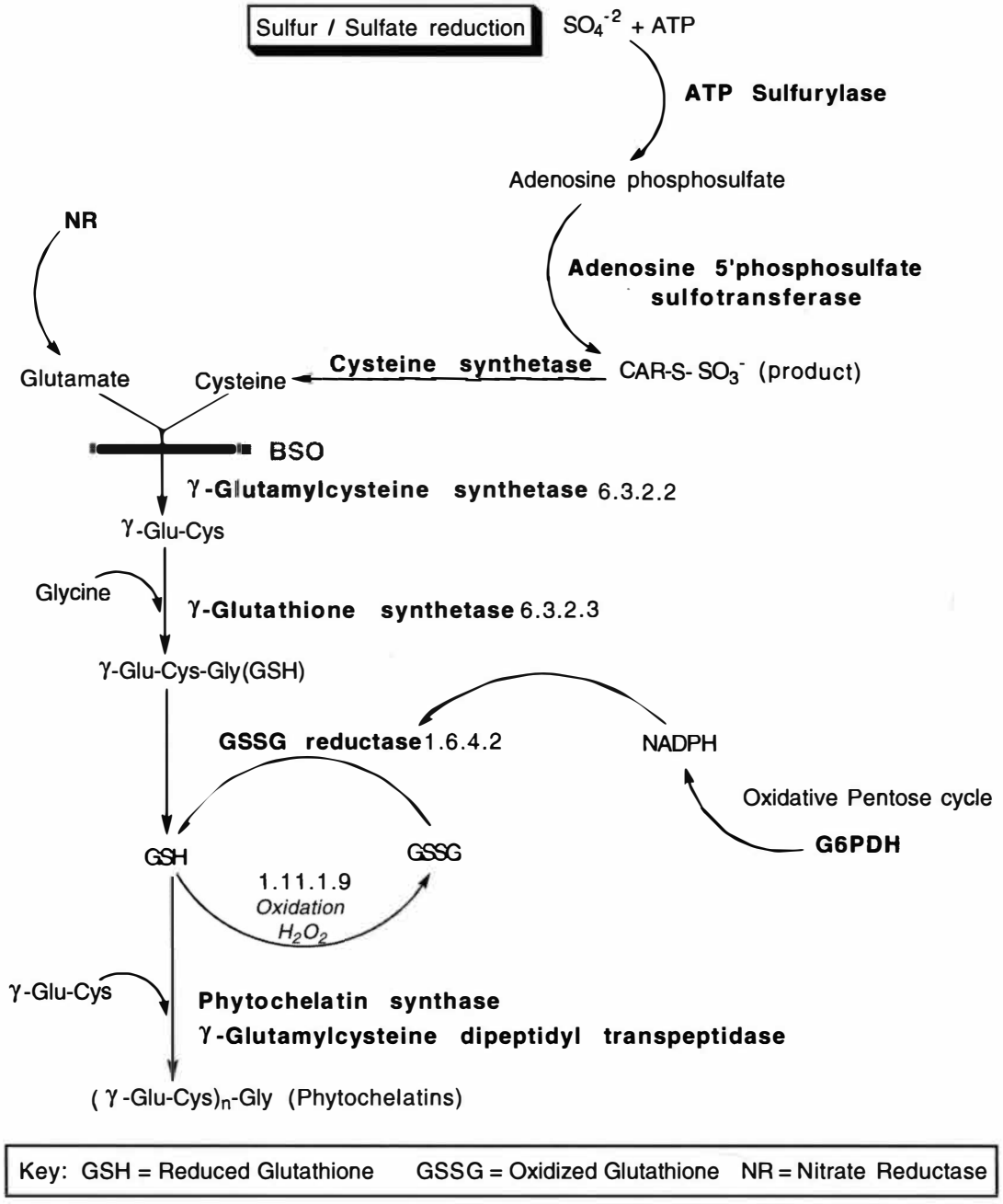


Figure 4. Pathways Involved in the Biosynthesis of Glutathione and Phytochelatins. Glutathione biosynthesis is a two step process, where Glu and Cys are joined in the first step and Gly is joined in the second step. BSO (L-buthionine sulfoximine) is also shown here for its use as an inhibitor of the first step of glutathione biosynthesis. Phytochelatins are then produced by the transpeptidation of γ-Glu-Cys moieties onto another glutathione molecule.

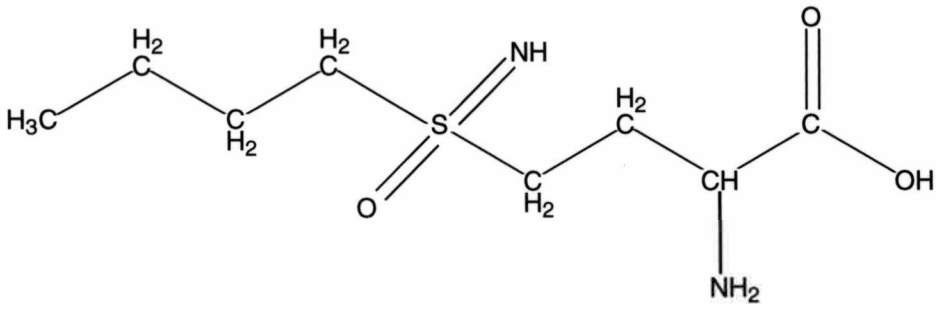


Figure 5. Chemical Structure of L-Buthionine Sulfoximine (BSO). CAS Registry Number 83730-53-4. Molecular Formula: $C_8H_{18}N_2O_3S$ FW=222.3.

Arabidopsis as a Model Organism

Research and Arabidopsis

To explore plant responses to heavy metal contaminants, the model organism *Arabidopsis thaliana* (L.) Heynh (var. Langsberg erecta) has been chosen. *A. thaliana* is a small winter annual crucifer, used in research since the early 1900's because of its small size, rapid life cycle (45-60 days), and high seed yield. *Arabidopsis* also has a small chromosome number(2n=10), no indigenous transposons, and a small genome ($1X10^8$ bp, the smallest known among angiosperms). Together, these reasons help to explain the current world efforts to map its genome. The ability to produce phytochelatins and its tolerance to Cd (typically to concentrations near 100 μM) has also made *Arabidopsis* the model of choice for heavy metal studies (Koncz et al., 1992). Commonly called the thale cress, *Arabidopsis* is a member of the Brassicaceae and has been extensively used in plant research (Redei, 1975). Isolation of morphological and physiological mutants can be done with ease.

Isolation of Mutants Tolerant to Heavy Metals

Isolation of mutants have been done by using root growth as a tolerance index to heavy metal toxicity. Recently, a rapid screening method was developed using a vertical mesh transfer system (VMT) which allows for the isolation for metal-tolerant mutants in about 5 days (Murphy & Taiz, 1995). Copper sensitive mutants have been successfully isolated by Murphy and Taiz (1995) using the VMT system, although this technique has not been used to isolate hypertolerant mutants of *Arabidopsis thaliana*. The VMT system also is convenient because it allows a high number of seeds to be screened (35,000 per tank) as well as the study of physiological parameters such as dose-response curves (Murphy & Taiz, 1995). Furthermore, phytochelatins are known to be produced in *A. thaliana* upon exposure to Cd and a few Cd sensitive mutants have already been isolated and characterized (Howden & Cobbett, 1992; Howden et al., 1995a; Howden et al., 1995b). Some mutants have been characterized as deficient in the production of glutathione while others are phytochelatin deficient, suggesting that mutations of a single component in the phytochelatin biosynthetic pathway can have a negative or positive effect on the ability to cope with heavy metal toxicity. In addition, tolerance levels for wild type plants have been established previously using the VMT system. *A. thaliana*, ecotype Langsberg erecta, has a tolerance to 90 μM $CdCl_2$ (Murphy & Taiz, 1995).

Purpose of the Study

The objective of this study was to isolate hypertolerant mutant plants of *A. thaliana* that can be used to elucidate mechanisms of elevated metal accumulation. For this purpose, the following hypothesis and predictions were formulated:

1. Can Cd hypertolerant mutants of *A. thaliana* be isolated using the VMT

system? If Cd hypertolerant mutants can be isolated using the VMT, then seedlings will continue to grow when exposed to elevated Cd levels (>90 μM $CdCl_2$).

2. Is the hypertolerant phenotype an inheritable trait and is it inherited as a single Mendelian locus? If this trait is inheritable, M3 and M4 progeny of the mutant line(s) will also exhibit the tolerant phenotype. If the hypertolerant trait is inherited in a Mendelian fashion, wild type x mutant line crosses will produce F1 and F2 progenies that follow the expected result of a monohybrid cross.

3. Can these hypertolerant mutants accumulate elevated levels of Cd? If the hypertolerant mutants are hyperaccumulators of Cd, then high levels of endogenous Cd will be observed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis of Cd treated plants.

4. Is Cd preferentially accumulated in the shoots? If metal allocation is enhanced in shoot tissues of mutant plant lines, elevated shoot levels of Cd will be observed by ICP-MS analysis of root and shoot tissues.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF CADMIUM-HYPERTOLERANT MUTANTS

Introduction

Heavy metal contaminants are a growing concern due to their toxic effects, persistence, and consequent agricultural losses. Cd uptake and storage in vacuoles by plants offers a method of "harvesting" contaminated soils by phytoremediation (McGrath et al., 1994). Phytoremediation has the potential of reclaiming contaminated soils as an alternative to traditional costly soil cleaning methods (Cornish et al., 1995). On the other hand, prevention of Cd entry into plant systems can be important in reducing bioaccumulation of heavy metals in the food chain and increasing agricultural yields. The isolation of plants hypertolerant to Cd can therefore provide a better understanding of the detoxification mechanisms involved with heavy metal exposure and has several potential commercial applications.

Previous studies into the role PCs have in the detoxification of heavy metals yielded mutant plants that are unable to accumulate Cd. Analysis of the mutations discovered two loci, *cad1* and *cad2*, responsible for the deficiency in phytochelatin and glutathione production respectively (Howden & Cobbett, 1992; Howden et al., 1995a). I initiated similar studies of hypertolerant mutant isolation to help elucidate important mechanisms of metal tolerance. The first purpose of this study was to isolate Cd hypertolerant mutants from *Arabidopsis thaliana* using a VMT system by exposing the plants to concentrations nearly twice the tolerance level determined for wild type plants (90 μM $CdCl_2$ (Murphy & Taiz, 1995)). Secondly, I wished to establish whether the hypertolerance trait of isolated putative mutants is

inheritable and would be attributed to the exclusion or bioaccumulation of Cd. Since the ultimate goal of this research is to grow mutants on Cd contaminated soils; germination, flowering, and silique numbers were studied in the presence of Cd. Furthermore, BSO was used to evaluate the possible role of PCs in the hypertolerance of the isolated mutants. This was done in an attempt to provide a biochemical characterization of the mutation(s).

Materials and Methods

Seed Surface Sterilization

Large Scale Sterilization

Wild type and ethyl methyl sulfonate (EMS) mutagenized seeds of *Arabidopsis thaliana* of the ecotype Langsberg erecta were purchased from Lehle Seeds Co. (Round Rock, Texas) and surface sterilized according to the distributors instructions. Approximately 2000 seeds were slowly added to Falcon tubes containing 20 ml of sterile water and 0.1% (v/v) Triton X-100 and vortexed for 1 minute. The solution was placed in a shaker for 20 minutes, after which it was vortexed for 1 minute and returned to the shaker for an additional 20 minutes. At this point, any seeds adhering to the tube walls and any floating debris were removed. The Triton solution was carefully decanted and the remaining seeds were washed with 95% ethanol (EtOH) containing 0.1% (v/v) Triton X-100. The EtOH-seed solution was vortexed for 1 minutes and then placed on to the shaker for 3 minutes. The seeds were allowed to settle for 1 minute, EtOH solution decanted and replaced with 30% Chlorox solution (v/v) containing 0.1% (v/v) Triton X-100. The seed-Chlorox solution was vortexed for one minute and then shaken for 8 minutes. Following shaking, the

seeds were allowed to settle for 1 minute and then the Chlorox solution was decanted. The seeds were then rinsed 5 times with 20 ml sterile water (2 minutes per rinse).

Small Scale Sterilization for Germination Experiments

Arabidopsis thaliana seeds from isolated mutants and the wild type seeds were used. Approximately 1000 wild type or M4 mutant line seeds were added to 4 well cell culture plates followed by 1 ml of sterile water containing 0.1% (v/v) Triton X-100. The seeds were drawn up and expelled 5 times using a micropipette and placed onto a shaker for 20 minutes, after which they were mixed again using the micropipette and returned to the shaker for another 20 minutes. Pipette tips were changed each time a new plant line or solution was used to avoid cross contamination. The Triton solution was then removed and replaced with 95% ethanol (EtOH) containing 0.1% (v/v) Triton X-100. The EtOH solution and seeds were mixed using a micropipette and then shaken for 5 minutes. The EtOH solution was removed and replaced with 30% Chlorox solution (v/v) containing 0.1% (v/v) Triton X-100. The Chlorox solution and seeds were mixed using the micropipette and then placed onto a shaker at 45 rpm for 9 minutes. Following shaking, the seeds were rinsed 5 times with 1 ml of sterile water (1 minute per rinse). A small quantity of water was left in the wells to prevent clumping of the seeds. Sterile seeds were transferred to the appropriate Petri dishes using a sterile tooth pick or a micropipette. Approximately 3000 seeds per plant line, 30-40 seeds per replicate (n=9), were used to asses germination rates at concentrations ranging from 0 to 200 μM $CdCl_2$ in 20 μM $CdCl_2$ increments.

Isolation of Mutants

Vertical Mesh Transfer System

Tank Setup. As described by Murphy and Taiz (1995), a small scale Vertical Mesh Transfer (VMT) system consisting of single strength glass squares (9.5 cm x 9.5 cm x 3 mm thick) was constructed. The miniplate tank system was contained in a plastic growing tray (20 cm^2) covered by a clear plastic lid (Jiffy, Batavia, IL; 246-11). The assembled glass plates were mounted on test tube racks, holding up to 20 plates with approximately 200 - 300 seeds per plate, and placed under continuous fluorescent light of 150 μmoles m^{-2} s^{-1} (measured using PMA2100 detector; Solar Light Co.). 1.6 L of 0.25 X Murashige-Skoog (MS) (Sigma Chemical Co.; M5524) medium in 1mM 2-[N-N morpholine] ethanesulfonic acid (MES) at pH 5.5 was added to each growth tray and sealed with parafilm to prevent contamination and evaporation losses (Figure 6).

Plate Assembly and Screening of Seedlings. Glass plates were assembled in tanks with a square of 3MM chromatography paper (9.5 cm^2) (Fisher Scientific) saturated with growth medium, and a square of polyamide nylon mesh (Tetko Inc.; Nitex® 3-15) the same size as the glass plate. The plate assembly was placed at a slight angle with sufficient growth medium to cover the bottom of the plates to a depth of 0.5 cm.

The plates were prepared by placing a clean glass plate on a plastic micropipette tip box inside a tray (Note - to avoid contamination, the MS solution, glass plates, 3MM paper, nylon mesh, and test tube racks were all autoclaved. The work surface was wiped with 70% EtOH and latex gloves were used). A single 3MM paper square was placed on top of the glass plate and saturated with 0.25 X MS

containing 1mM MES (Sigma Chemical Co.) at pH 5.5. To avoid any air bubbles and wrinkles, a sterile glass pasteur pipette was rolled over the chromatography paper. A sheet of Nitex® polyamide nylon mesh was then placed on top of the chromatography paper and rolled flat with a glass pipette. Additional 0.25 X MS solution was added to ensure the surface was thoroughly wet. Excess solution was removed by holding the plate vertically to drain. Seeds were sprinkled evenly over the mesh by shaking a 1.5 ml microcentrifuge tube containing holes for a final density. The seeded plates were placed into the growing tray containing 0.25 X MS solution, covered with a lid, and placed under the light for 72 hours.

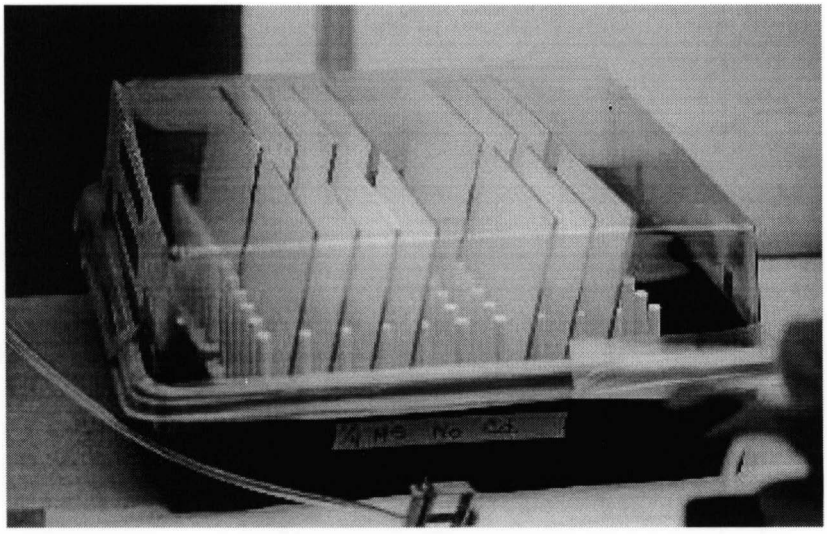


Figure 6. VMT Setup Containing Mini Glass Plate Assemblies.

After the initial 72 hour incubation, the seeded mesh was then lifted and transferred to a second paper-glass assembly saturated with 0.25 X MS. The mesh was rotated 90 ° from its original orientation so that the emerging roots were parallel to the bottom of the growing tray. Seedlings were incubated for an additional incubation period of 36 hours. At the end of this second stage, the roots of seedlings typically grow an additional 2-5 mm downward and form a distinct right angle.

Seedlings that did not form the first right angle turn were eliminated from the screen as they represented mutations unrelated to metal toxicity or wild type variants (Murphy & Taiz, 1995).

The mesh and seedlings were transferred a second time to a new paper-glass assembly soaked in 0.25 X MS solution containing 200 μM $CdCl_2$, and rotated 90°. The seedlings were covered and placed under the light for a further 36 hours. Following the final incubation, seedlings that formed a second root right angle and grew 2 mm or more were selected and presumed to be putatively hypertolerant to 200 μM Cd. Seedlings that failed to form a second right angle were considered to be Cd-sensitive.

The selected M2 mutant plants were transferred into pots containing moist soil. Pots were covered with clear plastic wrap to increase humidity for the first week. During this period, M2 seedlings were acclimated to the air humidity gradually by placing holes in the plastic wrap. M2 seedlings were finally transplanted into individual pots to allow self-fertilization and the production of a seed bank of M3 seeds.

Genetic Crosses

The seeds obtained (M3 seeds) from the isolated M2 seedlings were germinated (M3 plants) and then self-crossed to obtain M4 seeds (Figure 7). M4 seeds were used as a seed bank from which all subsequent experiments were performed. For the parental cross, the putative hypertolerant *cdht1* and *cdht4* M4 mutant seeds were germinated and the resulting M4 plants were crossed with wild type plants by hand pollination to obtain an F1 generation. Reciprocal crosses were performed to avoid any phenotype variations that may occur due to maternal

influences. The isolated F1 and F2 seeds were exposed to 200 μM $CdCl_2$ using the VMT system to record the resulting phenotype of the generation.

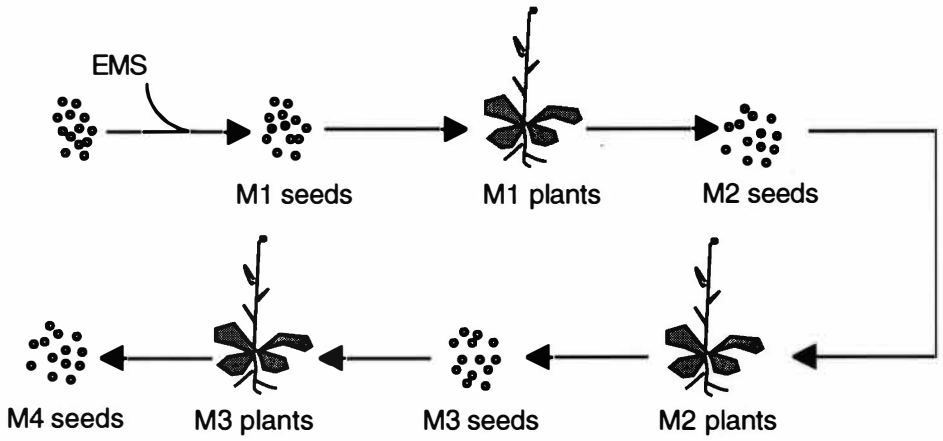


Figure 7. EMS Mutagenesis and Seed Bank Generation. Wild type seeds were exposed to EMS to generate random mutations. The mutagenized seeds were grown and selfed to produce M2 seeds. EMS is a base analog and causes point mutations. Self pollination allows for the production of homozygous seeds and the isolation of recessive phenotypes by visual inspection of M2 mutants. M3 seeds were grown to produce a seed bank.

In order to perform the genetic crosses, all open flowers of the receiving plant and immature flower buds were first removed to avoid any unwanted self-fertilized crosses. The remaining three to four buds, which were close to opening, were left devoid of petals, sepals, and anthers with the aid of a forcep and magnifying lens or microscope. Anthers were inspected under the microscope prior to their removal to see if mature pollen has already been produced. Buds that containing mature pollen were discarded. A mature flower was then removed from the pollen donor plant and brushed several times against the pistil of the receiving flower. Success of the pollination was evident after 24 hours. Once the siliques were formed and mature, the harvested seeds were dried in an oven at 37 °C for 3 days and later

used for subsequent back crosses (F1 x F1). The harvested seeds were used to test for Cd tolerance to 200 μM $CdCl_2$ as mentioned above and scored. VMT Cd-tolerance results were then compared to the predicted results for a monohybrid cross, to determine if the Cd tolerant traits were carried in a single locus and inherited in a Mendelian fashion (Figure 8).

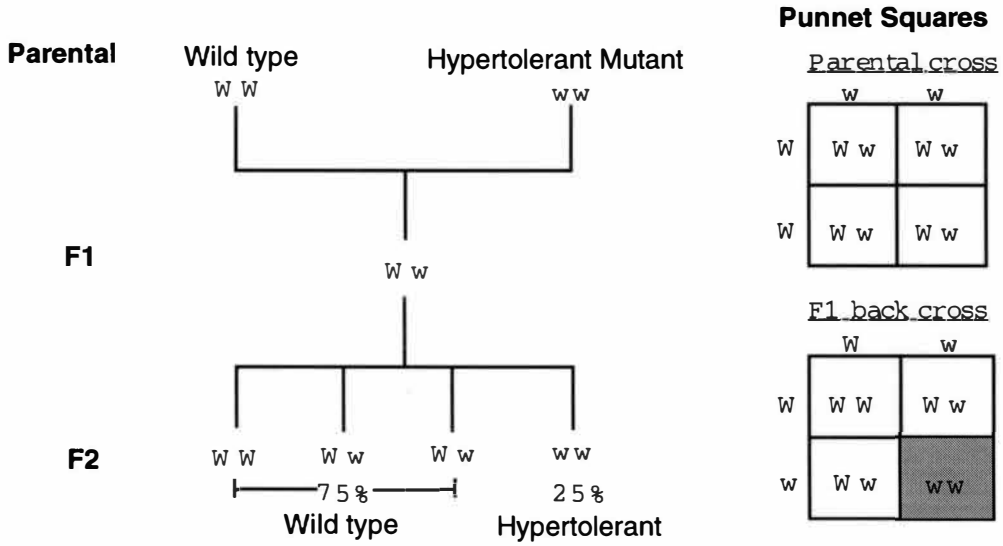


Figure 8. Representation of Predicted Results For a Monohybrid Cross.

Physiological Response Studies to Cadmium Exposure

Arabipatch Soil Preparation

Arabipatch (Lehle Seed Co.) was selected as a growing medium, as it provides optimal growth for *Arabidopsis* in soil. Arabipatch is composed of a sphagnum peat moss, bark, dolomite, and vermiculite mixture. Arabipatch soil medium was heat-treated (180 °F, 4 hours) and controlled release fertilizer (Lehle Seed Co.; PM01, osmocote) was added at a rate of 727 mg L^{-1} of dry soil. Seeds were sown and grown according to the manufacturer recommendations.

Seed Germination Experiments

Wild type and *cdht1* and *cdht4* mutant lines were investigated for their ability to germinate under various Cd concentrations. Sterile petri dish assemblies (Lab Teck, 100 X 15 mm) containing sterile filter paper (Sigma Chemical Co.; ashless #40) and 900 µl 0.25 X MS with 1mM MES (Sigma Chemical Co.; M5524) at pH 5.5 were divided into two sections. Plates were augmented with the appropriate Cd concentration, ranging from 0 to 200 µM $CdCl_2$ in 20 µM increments. Wild type and *cdht1*, *cdht2*, *cdht3*, *cdht4*, *cdht5*, and *cdht6* seeds were sown onto the filter paper, dispensing similar number of wild type and mutant seeds on a separate dish section, and placed under fluorescent light (16 light / 8 dark photoperiod) at room temperature. After a period of six days, the number of germinated seeds was recorded.

Flowering and Silique Formation

Seeds of wild type and (M4) *cdht1* and *cdht4* mutant plant lines were sown onto moist Arabipatch soil, placed under fluorescent light (16/8 light to dark photoperiod) at room temperature and watered by sub-irrigation with distilled water. Three weeks after germination, pots were watered by sub-irrigation with 0 or 75 µM $CdCl_2$ solutions containing 1 mM MES at pH 5.5 as needed. Formation of buds, flowers and silique production were recorded during the following 5 weeks.

Cadmium Uptake and ICP-MS Analysis

Experimental Setup

Seeds of wild type and (M4) *cdht1* and *cdht4* mutant plant lines were planted

onto plastic containers (20 X 20 cm, Jiffy) containing 16 square cavities in groups of four (Figure 9). Felt wicks were placed into inserts and then filled with Arabipatch soil medium, leaving at least one square open to allow watering by sub-irrigation. Seeds were suspended in 1% agar, sown, and placed in growth chambers at 24/20 °C and 16/8 (light/dark) photoperiod with a photon flux density of 200 μmoles m^{-2} s^{-1} (measured using PMA2100 detector; Solar Light Co.). Seedlings were thinned out the second week after germination to a density of ten to twelve seedlings per cavity. During the fourth and fifth weeks after sowing, seedlings were treated twice with 500 ml solutions containing 0, 75, or 150 μM $CdCl_2$ in 1 mM MES at pH 5.5. BSO treatments were also performed using 1mM BSO or 150 μM $CdCl_2$ plus 1mM BSO solutions containing 1 mM MES at pH 5.5. The experimental Cd treatments, 75 and 150 μM $CdCl_2$, were selected to encompass the tolerance and toxic index levels described by the VMT system.

Plants were harvested 42 days after being sown. All of the leaves were removed from plants and dried in coin envelopes for two days at 55 °C. Roots from each replicate were harvested by washing the soil out and rinsing the roots with distilled water prior to drying. Roots harvested were oven dried as above before ashing.

Reagents and Standards

Optima grade acids (Fisher Scientific) were used to prepare the ashing pre-treatment solution (H_2SO_4 diluted in Millipore grade water 1/1 v/v) and the ash solubilizing solution (HCl, HNO_3, and Millipore grade water 1/1/2 v/v/v). A set of standards were prepared from 1000 ppm Cd Standards (Inorganic Ventures) by serial dilution using 2.5% HCl and 2.5% HNO_3 to reproduce the sample matrix.

Blanks were prepared using 2.5% HCl and 2.5% HNO_3 in Millipore grade water and spiked with Rhodium (Rh) (Inorganic Ventures) internal standard. Standard solutions were also spiked with Rh.

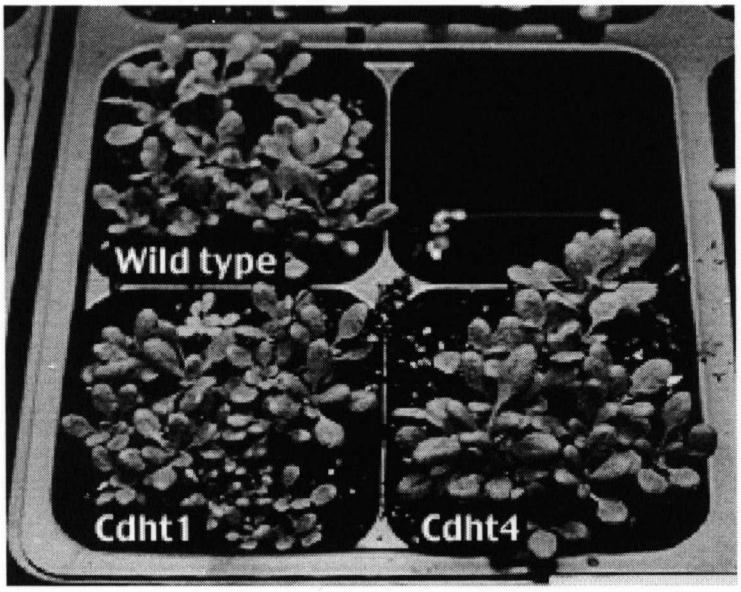


Figure 9. Growth Tray With Seedlings on Three Cavities. Wild type seedlings and two of the isolated mutants, *cdht1* and *cdht4*, are depicted.

Reproducibility and percent recovery of the ashing protocol was also tested. Results were verified by spiking 200 mg of root and shoot tissues (n=10) with 1 ml of the 10 ppm Cd atomic absorption standard (Fisher Scientific), calcinated as described above, and analyzed by ICP-MS. Blanks were also used to test for cross contamination and Cd extraction from crucibles by ashing crucibles as described above, without the inclusion of plant tissue. Recovery from Cd spiked tissues was only achieved to 20 % ±9 (±RSE). Feinberg and Ducauze report recoveries of 90% ±10.6 (RSD), however in this study 8 to 40 times more sample tissue was utilized, the amount of Cd spiked was 2 to 4 times higher, and the type of crucible used was

high purity quartz. Therefore, this may account for the higher recovery.

Tissue Calcination

Ceramic crucibles were cleaned by a 1 hour rinse in an ashing solubilizing solution (ACS Reagent Grade acids), rinsed 3 times with Millipore grade water, and dried. Dried plant material was now ground, weighed, and placed into a crucible. The tissue was treated with 2 ml of pre-ashing solution (H_2SO_4 Optima grade diluted in Millipore grade water 1/1 v/v), covered, and calcinated at 750°C for a period of 12 hours in a muffle furnace (Fisher Scientific). The ashes were dissolved in 2 ml of solubilizing solution (as mentioned above, using Optima grade acids), followed by a single rinsing of the crucible with Millipore grade water to dilute the sample to a final volume of 10 ml. To each solution, a spike of Rhodium was added as an internal standard to correct for variations in the signal, caused by signal drift and matrix effects, measured by the Inductively Coupled Plasma source Mass Spectrometer (ICP-MS) over time. Tissue samples were then filtered using a 0.45 μm polyethylene filter (Scientific Resources Inc.) and stored in volume scintillation vials with polyethylene lined caps, used to prevent sample loss by evaporation and to prevent the reaction of acids with the cap lining. This protocol was adapted from Feinberg and Ducauze (1980).

ICP-MS Analysis

ICP-MS determinations were performed using a Perkin-Elmer Sciex Elan 250 with the 5000 upgrade. The ICP-MS was calibrated daily using a solution of Magnesium (Mg), Rhodium (Rh), and Lead (Pb) (10 ppb each), and scanning at the following mass charge ratios ($^{m}/_{z}$): 24 for Mg, 103 for Rh, 207 and 208 for Pb,

and 220 for background noise. Cd concentrations were determined using the Elan 5000 software by subtracting the blank intensity and measuring the ion intensity of Cd ($^{m}/_{z}$ = 114), corrected by the ion intensity of Rh ($^{m}/_{z}$ = 103). Detection accuracy was tested by running a 100 ppb standard after every 10 samples. Detection limits for Cd on an ICP-MS have been calculated to 0.1 ppb. The instrument detection settings and nebulizer type used are described in Table 1.

Table 1

ICP-MS Instrument Settings Used for Semi-Quantitative Analyses

Elan 250 (5000 upgrade) ICP-MS (Perkin Elmer Sciex)	
Option	Setting
Resolution	Normal - 1 mass unit
Measurements/peak	3
Scanning mode	Peak hopping
Points/spectral peak	3
Sweeps/reading	1
Readings/replicate	3
Replicates	3
Replicate time	600 milli seconds
Dwell time	200 milli seconds
Noise level	blank subtracted
Sample read delay	90 seconds
Analysis time	17 seconds
Plasma power	1.1 KW

Table 1—Continued

Elan 250 (5000 upgrade) ICP-MS (Perkin Elmer Sciex)	
Option	Setting
Nebulizer type	TR-30-C3
Nebulizer flow	optimized daily
Plasma flow	13 L/minute
Auxiliary flow	1.5 L/minute
Option	Setting
Lens voltages	typical
E1	91
B	28
S2	57
P	31

Effects of BSO and MES on the Extractability of Cadmium From Soils

Soil Analysis for Total Recoverable Analytes

Determination of the total Cd content in Arabipatch soil was performed using the EPA method 200.8 (EPA, 1994). Standards were prepared as described above in 2.5% HCl and 2.5% HNO_3. A representative sample of the Arabipatch soil was homogenized using a mortar and pestle and sieved through a 20-mesh copper sieve. From the ground material, soil was mixed, weighed in 1.0 ± 0.01 g aliquots and transferred into 250 ml beakers for acid extraction. Four ml of 1:1 Millipore (18 MΩ) H_2O:HNO_3 (v/v) and 10 ml of 4:1 Millipore H_2O:HCl (v/v) were added to each

beaker and covered with a watch glass. Extraction then proceeded by gentle reflux of the solution on a hot plate, adjusted to maintain a temperature of 85°C while avoiding vigorous boiling. After 30 minutes of reflux, the samples were allowed to cool before transferring the sample into 100 ml volumetric flasks. The samples were then diluted to 100 ml with Millipore grade water, mixed, and allowed to settle overnight. Analysis was performed on ICP-MS by transferring 50 ml of sample into polypropylene bottles and spiking them with Rh to a final concentration of 50 ppb.

Soil Extraction of Cadmium by BSO and MES

Possible BSO interactions with Cd solubility in the soil were also investigated. Due to the low background levels of Cd in the Arabipatch soil (215 ±18 ng Cd g^{-1} dry weight (±SE)), interactions were studied by spiking soil with Cd prior to the addition of BSO.

A representative sample of soil was oven dried to determine the percent solids present. The dried soil sample was homogenized as before, spiked with Cd to provide a final concentration of 800 ppb (using 1.2 mg Cd in 1 L of Millipore grade water to treat 180 g dry weight soil), and dried to constant weight using a round bottomed flask connected to a rotary evaporator set to a temperature of 55°C. After removing the soil from the flask, the sample was re-homogenized, mixed to ensure an even distribution of the Cd, and divided into 20 aliquots of 9 g for the various treatments.

Treatments were performed by adding 75 ml of 0, 1 mM, 1 μM, and 1 nM solutions of BSO in 1mM MES at pH 5.5 to 9 g of soil. The concentration of each BSO treatment was selected to overlap the range of Cd used under the Cd uptake experiments which were previously performed and cover 1:1 Cd:BSO (molar) concentrations. The soil was exposed to the treatment overnight at 23°C by revolving

the flasks in a rolling incubator.

Statistical Analysis

Chi square tests were performed on the F2 results from the genetic crosses to test the goodness of fit (α=0.5) to the expected values of a monohybrid cross. The remaining data analysis was performed using JMPIN statistical discovery software (1995) for Macintosh. Wilcoxon non-parametric tests were applied to evaluate germination rates because transformations (i.e $\sqrt{\arcsin}$, ln, and square root) failed to achieve a normal distribution. Pairwise comparisons on the accumulation of Cd in mutant and wild type plants was performed using the Tukey Kramers HSD test at the 0.05 significance level.

CHAPTER III

RESULTS

Mutant Isolation and Selection

From approximately 38,000 seeds tested, six putative hypertolerant mutants were successfully isolated from the VMT system and grown to produce seed. The isolated mutants were selected because root growth after exposure to 200 μM $CdCl_2$ was greater than 2 mm (Figure 10). These mutants were labeled *cdht1*, *cdht2*, cdht3, *cdht4*, *cdht5*, and *cdht6*. M3 and M4 seedlings of the isolated mutants did not differ in appearance from the wild type, when grown in the absence of Cd (see Figure 9). *Cdht1* mutants were slightly smaller in size when compared to the wild type, although no signs of chlorosis or changes in the growth habit were evident.

Germination Experiments

Based upon a series of germination experiments, only two mutants were selected for further investigation. The first mutant, *cdht1* (Cd hypertolerant 1), was chosen because it exhibited a higher germination rate compared to wild type seeds when exposed to Cd concentrations ranging from 20-200 μM (Figure 11). A second mutant was selected, *cdht4*, which had a lower germination rate compared to wild type under the same Cd concentration range as above. Mutants *cdht2*, *cdht3*, *cdht5*, and *cdht6* displayed LD_{50} levels below 60 μM $CdCl_2$ concentrations (data not shown) and were therefore not used for subsequent analysis. Wild type plants and *cdht1* and *cdht4* mutant lines had similar germination rates when grown in the absence of Cd, ranging between 85% and 93% germination (Figure 11). As seeds

were exposed to increasing Cd concentrations, wild type plants and all mutant lines exhibited a decrease in the rate of germination (Figure 11). *Cdht1* mutants had an LD_{50} greater than 200 μM $CdCl_2$, while Cdht4 and wild type seeds had an LD_{50} of 120 μM and 110 μM $CdCl_2$ respectively. Comparison of germination rates between wild type and *cdht1* mutants show a significant deviation between groups at the 80 to 200 μM $CdCl_2$ treatments, tested using Wilcoxon's non-parametric test. *Cdht4* mutants displayed germination rates similar to wild type and were not significantly different based on Wilcoxon's non-parametric test.

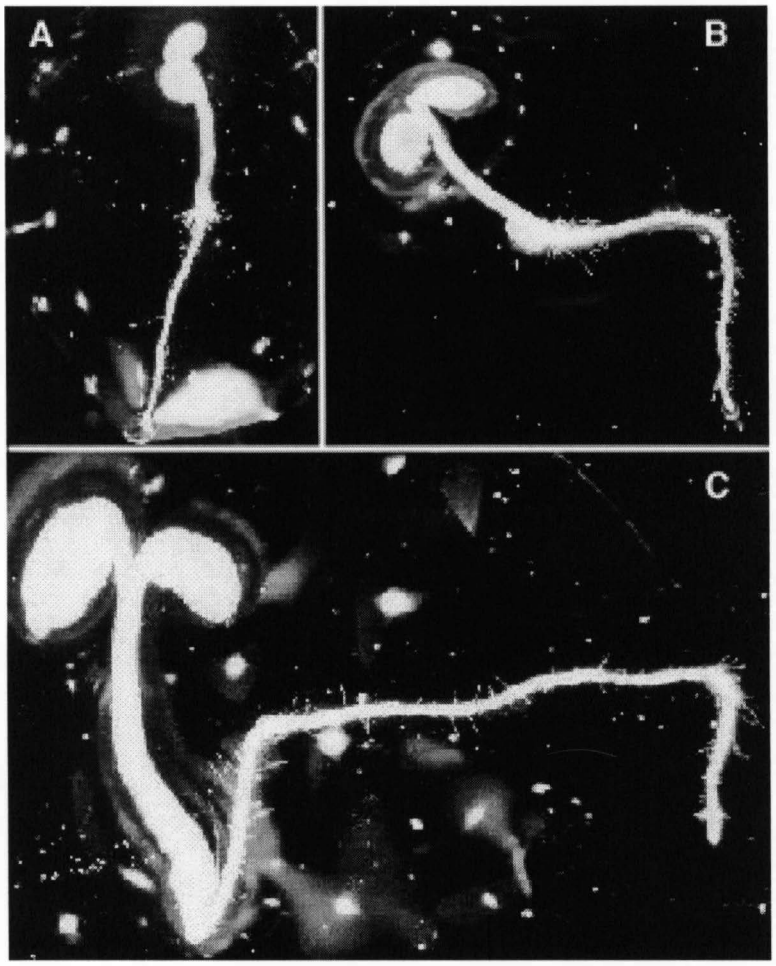


Figure 10. VMT Isolated Seedlings. After 6 days of growth, exposed to 200 μM $CdCl_2$ for 36 hours, seedling A was unable to form root bends, seedling B formed one root bend, and seedling C formed two root bends.

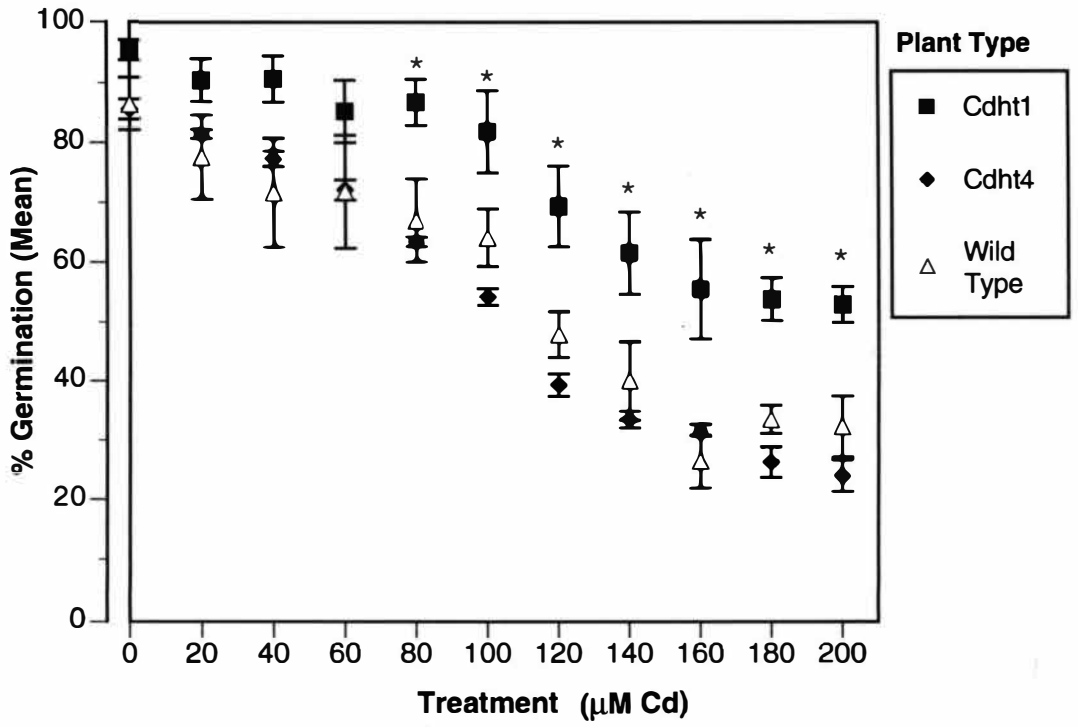


Figure 11. Comparison of Germination Rates. Wild type, cdht1, and *cdht4* seeds were exposed to Cd concentrations increasing by 20 μM. Values are the mean ±SE (n=9), scoring 30-50 seedlings per replicate. Statistical Analysis: * significantly different from wild type at 0.05, Wilcoxon non-parametric tests.

Genetic Crosses

In crosses of the *cdht1* mutant to the wild type, 107 out of 123 seedlings (87% tolerant) tested from the F_1 progeny exhibited the *cdht1* phenotype. The results obtained from the F_1 progeny VMT tests are similar to the results obtained for the parental generation for *cdht1* (M4 seeds), where 111 (88% tolerant) out of 127 seedlings displayed the tolerant phenotype. After self fertilization of F_1 plants, 327 of 446 seedlings (74%) of the F_2 progeny displayed the tolerant phenotype. Chi square test results for the expected values of a monohybrid cross (3:1 tolerant to sensitive ratio) yielded probability values falling between 0.5 and 0.7 (Table 2),

significantly supporting that the *cdht1* phenotype is inherited as a single dominant Mendelian locus.

Table 2

Phenotype Verification of Genetic Crosses Using the VMT System

Wild type X *Cdht1* Crosses						
Generation	n	% expected	% tolerant	df	X^2	p*
Parental Wild type	165	0	4			
Parental Cdht1	127	100	88			
F1 seedlings	123	100	87			
F2 seedlings	443	75	74	1	0.28	0.5-0.7

*=p>0.05 α (n=3).

Crosses of the *cdht4* mutant to the wild type yielded 8 out of 126 seedlings (6% tolerant) of the F_1 progeny with the *cdht4* phenotype. The results of the F_1 progeny are similar to the values obtained for the parental generation of the wild type (4% tolerant). After self fertilization of F_1 plants, 99 out of 429 seedlings tested (23% tolerant) from the F_2 progeny displayed the Cd hypertolerant phenotype. Chi square tests on the F_2 progeny supports that the *cdht4* hypertolerant character is inherited as a single recessive Mendelian locus (1:3 tolerant to sensitive ratio), with probability values falling between 0.3 and 0.5 (Table 3).

Table 3

Phenotype Verification of Genetic Crosses Using the VMT System

Wild type X *Cdht4* Crosses						
Generation	n	% expected	% tolerant	df	X^2	p*
Parental Wild type	165	0	4			
Parental Cdht4	94	100	85			
F1 seedlings	126	0	6			
F2 seedlings	429	25	23	1	0.8	0.3-0.5

*=p>0.05 α (n=3).

Flowering Formation

Observations on the flowering pattern of wild type and mutant plants show that untreated plants reach a maximum flower number 8 weeks after germination. Wild type and *cdht4* mutants produce an average of 4.7 flowers per plant, while *cdht1* only produces an average of 2.7 flowers per plant (Figure 12). Treatment with Cd reduced the number of flowers produced by the 8th week on all plant lines, an average of 2.4, 1.9, and 0.9 flowers were produced by wild type, *cdht1*, and cdht4, respectively (Figure 12).

Silique formation

Plants from the flowering experiments were also used to record the mean number of siliques produced by each plant, when treated with 0 or 75 μM $CdCl_2$

(Figure 13). Wild type and *cdht4* plants produced an average of 27 siliques, while *cdht1* plants yielded an average of 15 siliques per plant when Cd was not supplied. Exposure to 75 μM $CdCl_2$ reduced the mean yield of siliques to 22, 19, and 8 in wild type, *cdht1*, and *cdht4* plants, respectively. Wild type and *cdht4* plants appear to have similar silique production levels, when treated or untreated.

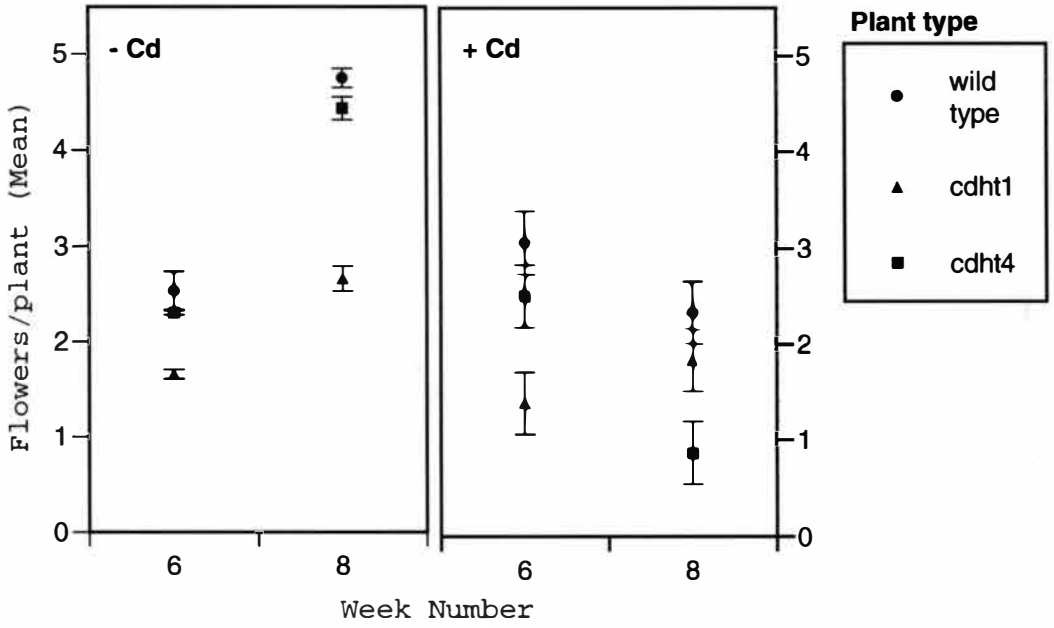


Figure 12. Effects of Cadmium on Flowering. Flowering patterns were monitored with plants exposed to 0 (-Cd) or 75 μM $CdCl_2$ (+Cd) over a period of 8 weeks. Week number represents the time point after germination. Data points are the mean of 20 plants ±SE.

Cadmium Accumulation

Total Cadmium Uptake

Experiments investigating the accumulation of Cd by wild type and mutant lines using ICP-MS indicate that plants not treated with Cd accumulate 45 μg Cd g^{-1}

dry tissue (Figure 14-A). Wild type plants treated with 75 μM $CdCl_2$ accumulate 542 μg Cd g^{-1} dry tissue, while *cdht1* and *cdht4* mutants accumulated 210 and 215 μg Cd g^{-1} dry tissue, respectively (Figure 14-B). Exposure to 150 μM $CdCl_2$ induced plants to accumulate 440, 191, and 191 μg Cd g^{-1} dry tissue for wild type, *cdht1*, and *cdht4* plants, respectively. All Cd-treated plants manifested signs of phytotoxicity to Cd by the presence of red pigmentation in the leaves, accelerated senescence, and chlorosis. Statistical analysis, using Tukey Kramer HSD (*= p>0.05), for variance show that *cdht1* and *cdht4* mutants accumulate significantly lower levels of Cd as compared to the wild type plants exposed to both 75 or 150 μM $CdCl_2$.

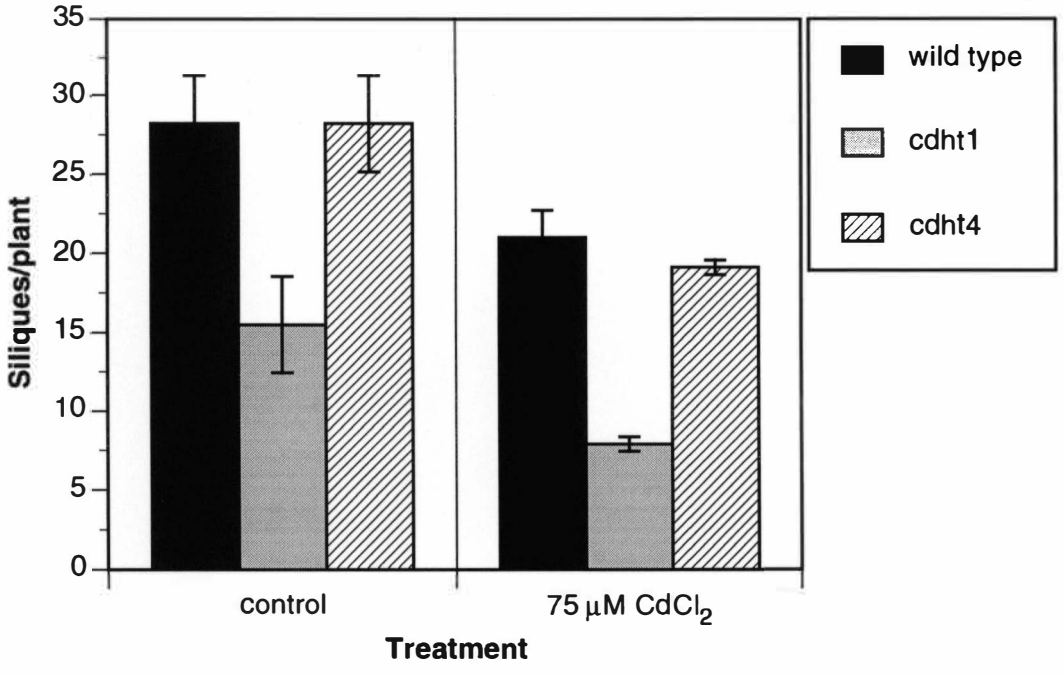


Figure 13. Effects of Cadmium on Silique Number. Plants were exposed to 0 or 75 μM $CdCl_2$ (N=20 ±SE).

Effects of BSO on Cadmium Uptake

Cd uptake by plants grown in unamended soil (i.e. no Cd addition and treated with BSO) was investigated. BSO caused a slight increase in the Cd levels in wild type and *cdht4* lines, however this was not significant (Figure 15-A, 15-C). BSO significantly elevated the endogenous Cd levels from 45 to 118 μg Cd g^{-1} dry tissue in *cdht1* mutants (Figure 15-B). No signs of toxicity, i.e. chlorosis or reduced growth, were visible in treated or untreated plants.

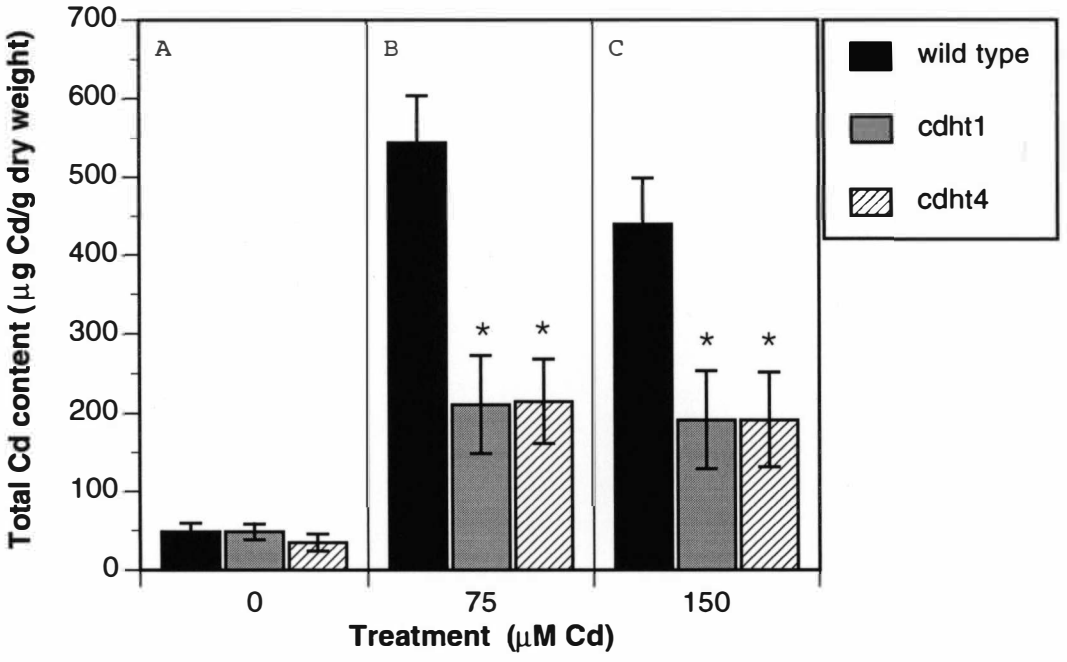


Figure 14. Total Cadmium Content Comparisons. Wild type plants and mutant lines were exposed to 0, 75, or 150 μM $CdCl_2$ for two weeks. Values are the mean ±SE of three independent experiments. Statistics: Comparison of all pairs using Tukey-Kramer HSD *=p >0.05.

When Cd was applied in conjunction with BSO, wild type and *cdht4* plants showed a slight reduction in Cd contents, but this was not significant (Figure 16-A, 16-C). In the *cdht1* line, Cd + BSO caused a slight increase in the tissue Cd level,

however this was not significant (Figure 16-B). Signs of phytotoxicity in 150 μM $CdCl_2$+BSO treatments appeared to be greater on all plant lines when compared to 150 μM $CdCl_2$. Comparisons of accumulation levels within a plant type and between treatments where not found to be significant using ANOVA (t-test and Tukey Kramer HSD at the 0.05 level).

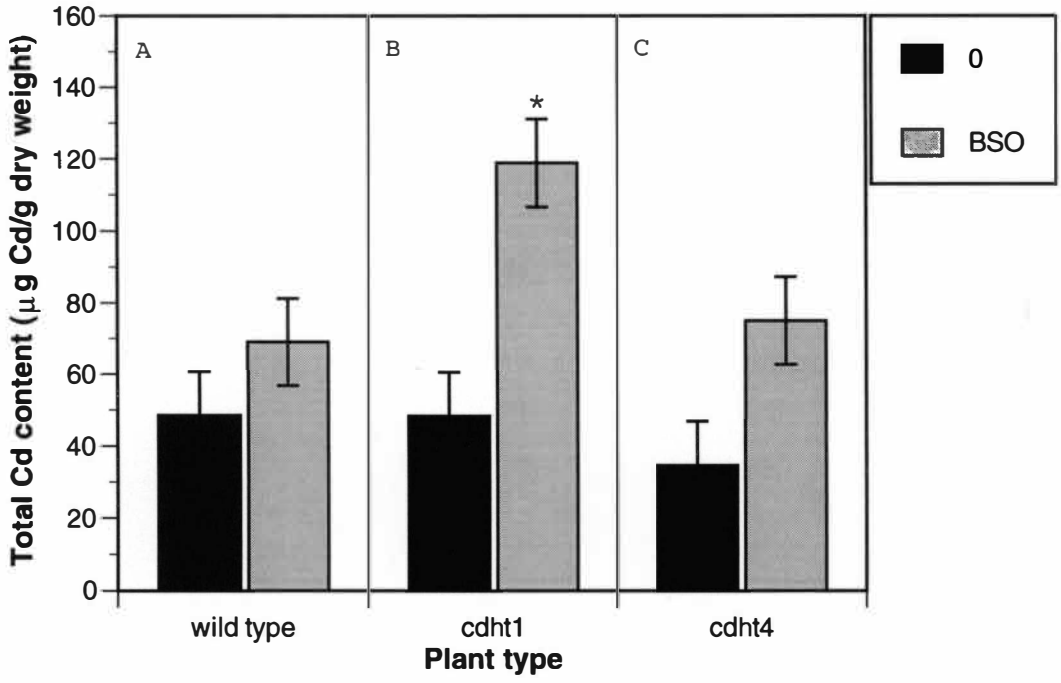


Figure 15. Total Cadmium Content Comparison in Plants Exposed to 0 μM $CdCl_2$ or 1mM BSO. Values are the mean ±SE (n=6). Statistics: Comparison of all pairs using Tukey-Kramer HSD * $p>0.05$.

Cadmium Root/Shoot Partitioning

Heavy metal partitioning within a plant was studied by comparing root and shoot levels of Cd (Figure 17 & Table 4). Under conditions of no added Cd, wild type, *cdht1*, and *cdht4* preferentially accumulated Cd in the shoots. The percent total Cd in

shoots was 60%, 81%, and 55% for wild type, *cdht1*, and *cdht4*, respectively. Wild type plants exposed to Cd preferentially accumulated more metal in the shoots, reaching 68% and 60% Cd for 75 and 150 μM $CdCl_2$ treatments, respectively. Under conditions of 75 μM $CdCl_2$, both *cdht1* and *cdht4* preferentially accumulated Cd in their shoot tissue. Percent total Cd in the shoot was 71% and 55%, respectively. However, under conditions of 150 μM $CdCl_2$, the trend seen at 75 μM $CdCl_2$ is reversed. At 150 μM $CdCl_2$, both *cdht1* and *cdht4* preferentially accumulated Cd in the roots tissue. The percent total Cd in shoot tissues was only 46% and 39%, respectively.

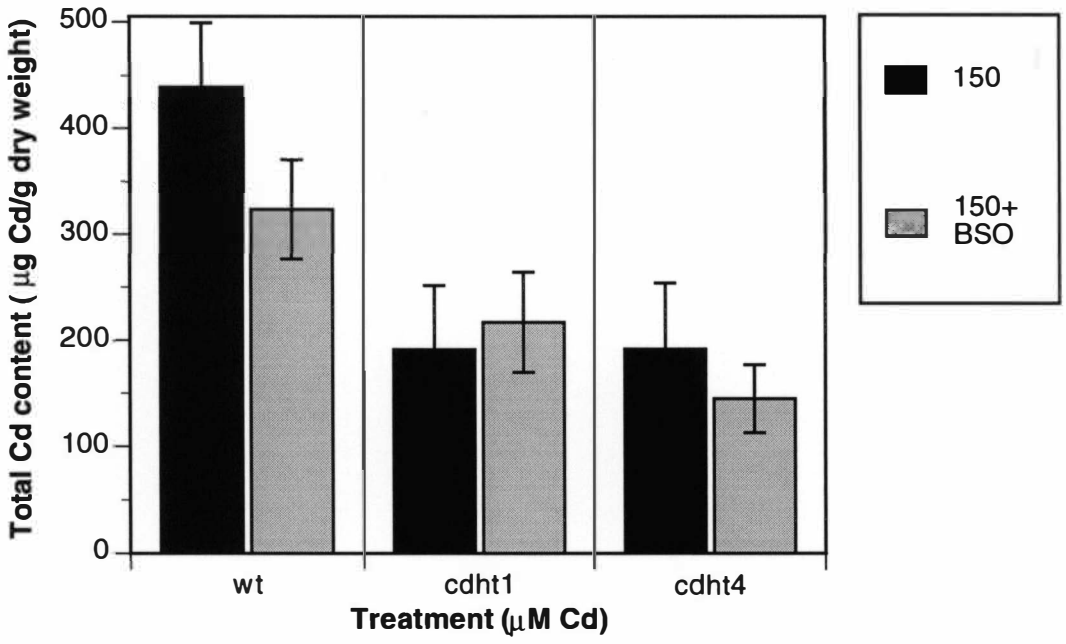


Figure 16. Total Cadmium Content Comparisons in Plants Exposed to 150 μM $CdCl_2$ or 150 μM $CdCl_2$ With 1mM BSO. Values are the mean ±SE of three independent experiments (n=9). Statistics: Comparison of all pairs using Tukey-Kramer HSD - non significant at the 0.05 level.

Comparisons of the root:shoot ratio of Cd accumulation were determined to

evaluate partitioning patterns. This provided additional insight into allocation pattern of Cd (Table 4). Table 4 expresses the data for Figure 17, with the addition of the BSO data, in the form of root:shoot accumulation ratios. Wild type plants consistently accumulate higher levels of Cd in shoots, regardless of treatment regime. In comparison, *cdht1* plants also have higher root:shoot accumulation ratios under all treatments, with the exception of the 150 μM $CdCl_2$ treated plants. *Cdht4* mutants also have higher root:shoot levels under all treatments, with the exception of 150 μM $CdCl_2$ and 150 μM $CdCl_2$ +BSO treatments. Both of the mutant lines tested appear to partition the Cd differently at 75 and 150 μM $CdCl_2$. In addition, the incorporation of BSO to 150 μM $CdCl_2$ treatments caused increases in shoot accumulation on all plant types, even though total Cd levels decreased (figure 16) for wild type and *cdht4*.

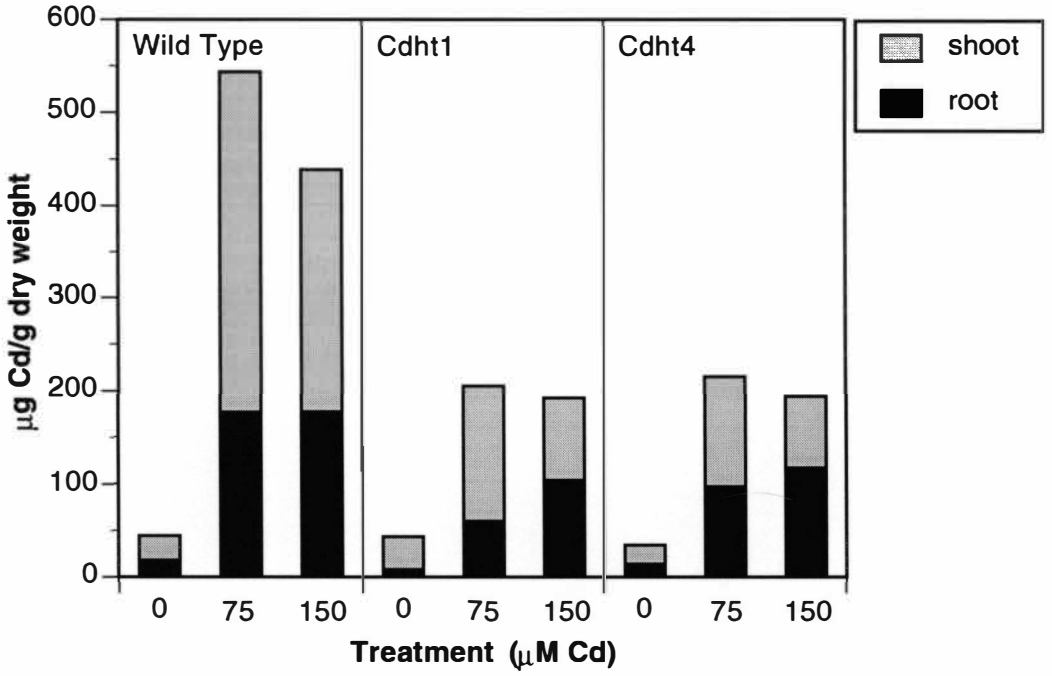


Figure 17. Comparison of Cd Accumulation in Shoot and Root Tissues. Plants were exposed to 0, 75, or 150 μM $CdCl_2$. Values are the mean of three independent experiments (n=9).

Soil Analysis

Soil analysis for total recoverable analytes determined the content of Cd in Arabipatch soil used in all experiments to be 215 ±18 ng Cd g^{-1} (±SE). Extraction of Cd from spiked soil samples was analyzed using ICP-MS (Table 5). Amount of Cd leeched out was below the detection limit of ICP-MS (0.1 ppb). The presence of 1 mM MES in all soil treatments had no effect on the leeching of Cd from soil. The method used to determine the total recoverable Cd from the soil used strong acids, which destroy organic matter and free Cd from soil constituents.

Table 4

Cadmium Allocation in *A. thaliana*

Root to Shoot Accumulation Ratios Under Various $CdCl_2$ Treatments					
Plant type	0 µM	75 µM	150 µM	BSO	150+BSO
Wild type	1:1.44	1:2.07	1:1.48	1:1.89	1:1.71
Cdht1	1:4.44	1:2.41	1:0.84	1:2.40	1:1.82
Cdht4	1:1.46	1:1.22	1:0.64	1:2.71	1:0.95

Ratios are the comparison of mean values (n≥6).

Table 5

Complexing and Extraction of Cadmium From Soil

Cadmium Release by MES or BSO + MES				
Sample	0 mM BSO	1 mM BSO	1 μM BSO	1 nM BSO
Soil	ND	ND	ND	ND

Data represents the mean for 5 samples per treatment. ND= below detection limit

CHAPTER IV

DISCUSSION

Cd is an environmental pollutant with great persistence and known to be toxic to animals, plants, microorganisms, and humans (Prasad, 1995; Robards & Worsfold, 1991). Efforts to cleanup and reclaim soils containing metals generally involved the use of conventional commercial systems, such as excavation, burial, and soil wash processing. These methods are expensive, can remove biological activity, and may alter the soil physical properties (McGrath et al., 1994). As an alternative, plants are being studied for their ability to bioaccumulate metals into shoots and stalks, offering a potential method of harvesting toxic elements. Phytoremediation, or the use of plants for remediation purposes, is a cost effective and aesthetic option which does not deleteriously alter soil physical properties. However, phytoremediation practices are limited by the number of tolerant species, their tolerance mechanisms, their root length, and biomass production levels. Research in many labs are beginning to address these limitations by isolating plants with altered ion nutrition, differential metabolism, and rooting structure (Cunningham et al., 1995). Analysis of these mutant plants can provide the genetic tools necessary to produce transgenic plants with increased phytoremediation potential. In addition, analysis of these mutations can provide insight into metabolic processes of detoxification in plants and help produce plants that reduce the entry of Cd into crops and the food chain. The purpose of this study was to isolate hypertolerant mutants that have altered physiological and genetic composition, which can be used to study key components of hyperaccumulation and exclusion mechanisms

involved in Cd tolerance.

Isolation of Mutants, Phenotype Verification, and Germination Response

During this study, six putative mutants were successfully isolated from the VMT system using root growth as an initial tolerance index to metal stress. The isolated mutant plants were grown to maturity and selfed to produce M3 seeds, and eventually a seed bank of M4 seed was produced for use in all experiments. The germination rate of wild type and putative mutants was investigated by exposure to 0-200 μM $CdCl_2$ concentrations in 20 μM increments. Observations on the rate of seed germination in mutants *cdht2*, *cdht3*, *cdht5*, and *cdht6* indicate that the mutation(s) present in these plant lines may also affect germination in the presence of Cd. Inhibition of germination by Cd has previously been reported in *Pinus resinosa*, where the release of Ca^{2+} from cell walls by Cd adsorption was thought to be responsible for the inhibition of germination (Strickland et al., 1979). Germination rates for wild type seeds and *cdht4* mutants have an LD_{50} of 110 and 120 μM $CdCl_2$, respectively, which closely agrees with the tolerance levels reported by Murphy & Taiz (1995) for this ecotype. In comparison, *cdht1* mutants were found here to have an LD_{50} above 200 μM $CdCl_2$ indicating that the mutation provides additional protection against Cd toxicity to the embryo during germination. Significant increases in germination rates occur at levels near the LD_{50} for wild type, where germination rates diverge for *cdht1* mutants. VMT tests on the M4 mutant generations further confirmed that the tolerance phenotype is an inheritable trait on *cdht4* and *cdht1* mutants, obtaining 85 and 88 % tolerance for each mutant, respectively (Table 2 and 3). The deviation from the expected 100% tolerant phenotypes may be attributed to processes related to VMT growth and scoring, where air bubbles, stringency selection, and distance from the nutrient solution may have

been influenced.

Genetic Crosses

Genetic crosses were performed to elucidate the pattern of inheritance for *cdht1* and *cdht4* mutants. The F_1 progeny from wild type X *cdht1* mutant crosses exhibited a tolerant phenotype, supporting the notion that Cd-hypertolerant phenotype is a dominant trait. Self pollination of the F_1 progeny were also performed, giving a 3:1 ratio of resistant to sensitive in the F_2 generation as expected for a single dominant Mendelian locus (Table 1). Analysis of the F_1 progeny obtained from wild type to *cdht4* mutant crosses exhibited a sensitive phenotype, supporting that the Cd-tolerant phenotype in this mutant is conferred by a recessive trait. The F_1 progeny was allowed to self pollinate, and the F_2 progeny gave a 3:1 sensitive to tolerant ratio expected for a single Mendelian recessive locus (Table 3).

Cadmium Uptake and Allocation

The capacity to accumulate Cd by wild type, *cdht1*, and *cdht4* plant lines was investigated in an attempt to further elucidate the tolerance mechanisms involved in these plants. Wild type plants were found to accumulate 2.6 and 2.3 times more Cd than *cdht1* and *cdht4* mutants exposed to 75 and 150 μM $CdCl_2$ treatments, respectively. Decreased accumulation of Cd was observed in both mutants, which rejects the hypothesis that Cd hypertolerance in both mutants is attributed to the hyperaccumulation of this metal. Furthermore, no significant differences in Cd accumulation are evident in plants grown in the absence of Cd, indicating that exclusion of Cd occured in a dose-dependent manner in these mutants. Treatments with 150 μM $CdCl_2$ did not result in increased accumulation of Cd compared to the 75 μM $CdCl_2$ treatments. Studies involving several plant species (cabbage and clover)

using different concentrations (Yang et al., 1995) produced a similar dose-dependent saturation response to influx, transport, and accumulation. Cabbage reached peak influx, transport, and shoot accumulation rates at 30 μM $CdCl_2$ while accumulation rates for roots peaked at concentrations near 10 μM $CdCl_2$ and then declined. Similar results were observed in Cd shoot:root ratios, where shoot tissue levels of Cd reached a maximum at 30 μM $CdCl_2$.

Cd accumulation studies were augmented by the use of BSO, a specific inhibitor of glutathione and phytochelatin synthesis, in an attempt to better characterize the biochemical nature of the mutations. Reductions in Cd accumulation were observed in wild type and mutant lines when 150 μM $CdCl_2$ treatments were supplemented with BSO. Although these differences were not statistically significant, this result suggests that phytochelatins may be important components of metal tolerance in the mutant lines (Figure 16). Other investigators have reported similar results in which BSO reduces Cd uptake but enhances nutritional deficiencies in Ca^{2+}, K^{+}, Mg^{2+}, and Mo^{3+} ions (Gussarson et al., 1996; Mendum et al., 1990). These results contradict the observations made for wild type, *cdht1*, and *cdht4* plant lines exposed to 0 μM $CdCl_2$ and BSO simultaneously. Under these conditions, Cd accumulation increased in all plant lines. Soil analysis and studies on the complexation ability of BSO and MES on the release of soil-bound Cd were undertaken in an attempt to explain increased Cd accumulation in the BSO treated plants. BSO has no effect on the release of soil-bound Cd (Table 5). Spiked Cd was never recovered by water soluble BSO and MES extraction. This may be due to Cd binding with humic and phobic acids found in the organic matter content of the Arabipatch soil (Manunza et al., 1995; Robards & Worsfold, 1991). Arabipatch soil is composed of materials such as sphagnum peat moss, dolomite, vermiculite, and bark, however, it has no "soil" (Personal Communication; Lehle, 1997). Interestingly,

the commercially available biosorbent BIO-FIX process uses sphagnum peat moss, algae, yeast, bacteria, and/or microbiota to remove inorganic pollutants such as Cd (Brierley, 1990). Moreover, a recent report on the enhanced accumulation of Cd by BSO in algal cell suspension cultures also contradicts the traditional metal response seen following BSO exposure (Cai et al., 1997). This suggests that BSO is capable of enhancing metal uptake, however, the mechanisms responsible is yet to be identified.

Tissue partitioning of Cd was also studied with the aim to determine allocation patterns of Cd in wild type and mutant plant lines. Root and shoot levels of Cd were compared for each plant line by calculating accumulation ratios (Table 4). Wild type plants accumulate 1.4-to 2-fold higher Cd levels in the shoots under all treatment regimes, probably due to consistent translocation rates. On the other hand, *cdht1* and *cdht4* mutant lines decrease their shoot levels of Cd with increasing exogenous Cd concentration. This indicates that Cd has an inhibitory effect on the uptake and allocation of Cd in the studied mutant lines. However, the mechanism responsible remains to be identified. Interestingly, enhanced shoot accumulation was observed under BSO and 150 μM $CdCl_2$ +BSO exposure in *cdht4* individuals, indicating that BSO is capable of enhancing Cd allocation into shoots.

Future Studies

Metal stress, heat shock, wounding and oxidative stress appear to have similar metabolic responses, suggesting that induction of tolerance mechanisms may be attributed to biochemical or biophysical signals shared among these stresses (Edelman et al., 1988; Gyorgyey et al., 1991; Cumming & Tomsett, 1992; Takahashi & Komeda, 1992). For example, heat shock proteins have been shown to be induced upon exposure to Cd while PCs are induced by a number of metals (i.e. Cd^{2+}, Cu^{2+}) (Wozny et al., 1990; Cumming & Tomsett, 1992; Prasad, 1995). PCs

have received considerable attention in the literature because they play a major role in metal detoxification in plants. Hyperaccumulators, excluders, and sensitive mutants for various metals are now being actively pursued and characterized in many labs, in an attempt to elucidate influx, translocation, and accumulation processes which occur during heavy metal tolerance (Zhang & Taylor, 1989; Howden & Cobbett, 1992; Howden et al., 1995a; Howden et al., 1995b; Murphy & Taiz, 1995; Wheeler, 1995; Chen et al., 1997). Many of these mutants exhibit altered ion nutrition strategies which increase or decrease ion uptake. For instance, Chen et al. (1997) have isolated Pb-tolerant mutants of *Arabidopsis* that do not significantly accumulate Pb^{2+} yet are able to hyperaccumulate Ca^{2+}, Al^{3+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, and Zn^{2+} via an undefined modification of uptake or translocation mechanisms. Translocation and uptake mechanisms altered in *cdht1* and *cdht4* mutant lines also remain to be characterized. Moreover, differential cation uptake has been observed in *Silene cucubalis*, where Cu^{2+} tolerance has been associated with reduced uptake due to altered transporter affinity (Lolkema & Vooijs, 1986). Metal ion uptake is thought to be regulated directly though differential uptake transporters or indirectly by signal transduction mechanisms induced by changes in cytosolic pH, transmembrane potential, and electrical currents (Wozny et al., 1990; Cumming & Tomsett, 1992). Evidence supporting the notion that changes in cytosolic pH may be a component in stress signaling mechanisms includes the induction of cAMP by cytoplasmic acidification (Caspani et al., 1985). Alterations in Ca^{2+} fluxes in the cell have also been proposed to be part of signal components in metal tolerance, although no direct evidence has been reported (Cumming & Tomsett, 1992),. Some evidence, however, has implicated the involvement of Ca^{2+} and calmodulin on Cd toxicity during germination of radish and pine seeds (Rivetta et al., 1997; Strickland et al., 1979). Studies on seed response to heavy metal toxicity, using

wild type and mutant lines such as *cdht1*, can help elucidate the proposed mechanism by which Cd inhibits germination.

The mutants, *cdht1* and *cdht4*, have altered patterns of Cd accumulation which remain to be characterized. It is interesting that inheritance patterns of both mutants is different. Additionally, mutant *cdht1* shows slight growth reduction and may be the result of nutritional deficiencies or energy costs brought on by its mechanism(s) of tolerance, perhaps through an energy-dependent metal efflux (Prasad, 1995). Metal uptake mechanisms remain unclear and further work is still needed to elucidate these processes and their role on metal tolerance. The use of Cd^{109} to investigate influx and translocation mechanisms in these mutants may help clarify these mechanisms of metal-tolerance. Possible signal transduction events involved in metal tolerance in these mutants still need investigation. The use of metabolism inhibitors (i.e. temperature), inhibitors and activators of calcium uptake/channels (i.e. EGTA and La^{3+}), and protonophores (i.e. dinitro phenol - DNP) would be of great use to clarify these questions. These mutants may provide valuable insights into the proposed roles signal transduction and gene regulation have on metal tolerance mechanisms while providing tools for reducing Cd entry into crops and increasing agricultural yields.

Conclusion

This study shows that the VMT system can be used to isolate Cd-hypertolerant mutants. Two mutant lines, *cdht1* and *cdht4*, were isolated and found to produce Cd-hypertolerant progeny. This supports the hypothesis that the hypertolerant phenotype is inheritable. Furthermore, genetic analysis confirms that the hypertolerant traits is inherited as a single Mendelian locus in *cdht1* and *cdht4* mutant lines, respectively. ICP-MS analysis of Cd uptake and allocation patterns in

tissues show that hypertolerance is conferred by exclusion, rather than by hyperaccumulation of Cd. Further analysis of *cdht1* and *cdht4* mutants should help elucidate the mechanisms involved in Cd tolerance.

Appendix A

List of Materials Used and Their Manufacturers

Table 6

List of Materials Used and Their Suppliers

Materials		
Item	Catalog #	Supplier
Arabipatch medium (soil)	PM-01	Lehle Seeds
Arabidopsis Seeds *L. erecta*	WT-4-1	Lehle Seeds
Arabidopsis Seeds *L. erecta* EMS	M2E-4-1	Lehle Seeds
Rhodeum ICP-MS Internal Standard	ICPMS-RH	Inorganic Ventures
BSO (L-Buthionine Sulfoximine)	B2515	Sigma Chemical Co.
Cadmium Chloride	C 2544	Sigma Chemical Co.
Cadmium Reference Solution	SC118-500	Fisher Scientific
Controlled Release Fertilizer	PM-11	Lehle Seeds
Filter Paper - Ashless Grade 40	Z24,123-7	Sigma Chemical Co.
Hydrochloric Acid (Optima Grade)	A466-250	Fisher Scientific
Isotemp Muffle Furnace 184A	10-550-185	Fisher Scientific
MES	M-8652	Sigma Chemical Co.
Murashige-Skoog Basic Salts	M5524	Sigma Chemical Co.
Nitric Acid (Optima Grade)	A467-250	Fisher Scientific
Polypropylene filters (Titan)	44525-PP	Scientific Resources Inc.
Plastic Growing Tray and Lid	246-11	Jiffy
Polyamide Nylon Mesh - Nitex®	3-15	Tetko Inc.
Sulfuric Acid (Optima Grade)	A468-250	Fisher Scientific
3MM Chromatography Paper	05-716-3R	Fisher Scientific

Appendix B

Manufacturer Contact Information

Table 7

List of Suppliers of Materials and Contact Information

Manufacturers and Suppliers

Company	Address	Phone Number
Fisher Scientific	1600 W. Glenlake Av., Itasca, IL 60143	(800)766-7000
Jiffy	Batavia, IL	(708)406-3900
Lehle Seeds	PO BOX 2366, Round Rock, TX 78680	(800)881-3945
Scientific Resources	1 Industrial Way, W.Eatontown, NJ 07724	(800)637-7948
Sigma Chemical Co.	P.O.Box 14508, St. Louis, MO 63178	(800)325-3010
Tetko Inc.	333 S. Highland Ave. Briarcliff Manor, NY	(914)941-7767

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