CADMIUM INTERACTIONS WITH THE ROOTS IN A FLOWING CULTURE SYSTEM AND SUBSEQUENT INFLUENCES ON XYLEM SAP THIOL RESPONSES OF *BRASSICA NAPUS*

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CADMIUM INTERACTIONS WITH THE ROOTS IN A FLOWING CULTURE SYSTEM AND SUBSEQUENT INFLUENCE ON XYLEM SAP THIOL RESPONSES OF *BRASSICA NAPUS*

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The research in this thesis was focused on cadmium (Cd) uptake and transport in *Brassica napus*, a plant with phytoremediation potential. Early efforts were targeted to development of a system to monitor Cd depletion by *B. napus* in hydroponic nutrient media. Termed the Experimental Cadmium Uptake Detection System (ECUDS), it was able to detect rapid Cd depletion in nutrient solution. ECUDS data suggested that biphasic depletion kinetics were occurring in the system, and therefore multiple mechanisms may have been present. One of these mechanisms was suspected to be apoplastic Cd binding, and thus a batch based system was developed to study these binding characteristics of *B. napus* roots. This system demonstrated that dead *B. napus* roots bound about 90% of the Cd, and suggested that apoplastic binding was responsible for significant Cd depletion in ECUDS. Temporal Cd binding data were further modeled using a kinetic equation derived from the law of mass action that assumed the binding would eventually reach equilibrium, and that there were a finite number of binding sites.

ECUDS was then reconfigured, using a larger volume to reduce the impact of apoplastic binding on system Cd depletion. Concurrently, xylem sap thiol response to Cd stress was investigated by measuring concentrations of cysteine, glutathione, γ-glutamylcysteine, and phytochelatin. These compounds were of interest since root-to-shoot heavy metal transport processes are poorly understood, yet maximizing these processes is vital for successful phytoremediation. Under Cd stress, xylem sap

concentrations of cysteine, phytochelatin, and a “glutathione-like” compound increased. These results suggested the likely involvement of phytochelatins and other thiol compounds in long distance Cd transport.

Cd depletion measurements indicated that apoplastic binding had less impact on Cd levels in the reconfigured system. Uptake processes were kinetically differentiable into a rapid and gradual phase in the Cd depletion profile. Plotting depletion rates from the gradual phase versus Cd loading concentration revealed a saturable response that was modeled using Michaelis-Menten kinetics. Thus, depletion measurement systems are capable of resolving uptake kinetics of high affinity (low K_m) transport systems and avoid the linear bias found in Cd uptake isotherms derived through desorption-based methods.

BIOGRAPHICAL SKETCH

Aaron James Saathoff was born in April 1976 in North Platte, Nebraska. As a young boy, he could be frequently found lifting up rocks and watching the millipedes, ants, pill bugs, and various other insects scramble for cover. Of course, collecting insects was the logical next step, which resulted in the occasional sting or bug bite as a reward for his efforts. Growing up, his parents frequently took him on summertime trips to various national parks in the U.S., which instilled in him a permanent awe and appreciation for nature. By the time he graduated from North Platte High School in 1994, his interests had turned from entomology to environmental issues. He attended the University of Nebraska-Lincoln, majoring in Environmental Studies with minors in Chemistry and Mathematics, and graduated with distinction in 1998. While an undergraduate, he started developing his laboratory skills and spent much of his early research career in laboratories at the Beadle Center for Biotechnology in Lincoln. By the time he was ready to pursue graduate studies, he was convinced of a need to work in an environmentally oriented field where he could utilize his skills in research, chemistry, and mathematics to focus on an applied research problem. This ultimately led him to the Department of Biological and Environmental Engineering at Cornell. While there, he met the love of his life, Elisa Salas, who was studying at the time for her D.V.M. They married in 2005, and he completed the requirements for his Ph.D. in 2008.

This work is dedicated to my wife, Elisa, for her unwavering support, and to my parents, Keith and Lupe, who instilled in me a lifetime love of learning

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Getting to this point—writing and defending a dissertation—is not an easy task, and certainly is not done without the help of numerous others. Indeed, graduate school can be immensely frustrating one day, then immensely rewarding on the next (those "Eureka" moments); the people we meet along the way make it possible to successfully navigate the twists and turns of the graduate student journey.

First and foremost, I would like to thank my advisor, Larry Walker, for supporting and providing advice about my research all along the way. He is the reason I initially came out to Cornell during my search for a graduate school, and I have not once regretted that decision during my time here. He has an uncanny knack for dealing with graduate students who are in the doldrums about their data and getting them to leave his office more inspired and ready keep moving forward on attacking the problem at hand. Perhaps most importantly, though, he has taught me the value of a good glass of French wine at the end of the day, and that what we do outside of the laboratory is just as important as what we do inside of it.

Next, I would like to thank my other committee members, Beth Ahner and Roger Spanswick, for their helpful discussions and technical expertise during the various stages of research. To Roger, whose help was especially appreciated in figuring out instrumentation issues during the early design stages and for always being ready to answer my numerous questions about plants, and to Beth, for her expertise in thiols, metal chemistry, and always being able to give me new ideas about how to tackle a problem, I offer my thanks.

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knew of which would get the job done, and he often had a funny comment to go along with it. Special thanks also go to Sue Fredenburg, who somehow manages to get the work done of 10 people with time to spare, and goes well above and beyond the call of duty when helping out graduate students. Our laboratory technician, Ed Evans, came on board during the later stages of my research; he was very efficient in getting supplies ordered that kept my research going, and was great at providing general help when needed. My officemates in the Walker research group (and the other students I met outside of it) also deserve thanks for the numerous discussions, technical, intellectual, political, or otherwise, that helped make the experience fun. I was lucky to have such a great group of people to work with. Outside of the office and laboratory, Trevor Gentry, David Toomey, and Kimberly Cleland are among many who made life in Ithaca a lot more enjoyable during my time at Cornell.

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1. INTRODUCTION

There is a need for environmentally friendly technologies that can be utilized to clean up soil and water supplies contaminated with heavy metals. Phytoremediation, the use of plants for such a purpose, is one such technology (Salt et al. 1998). Although still in its infancy, using plants for heavy metal extraction offers some potential benefits and solutions to persistent problems in environmental cleanup (Raskin 1996). For one, large, diffuse areas of pollution left over from previous industrial activity, termed brownfields[1], have resulted in areas of land that currently sit idle that could otherwise be put towards productive uses. Problematically, the cost of cleaning up these lands can exceed the market value of the land after redevelopment, which results in many sites simply remaining derelict. This problem is not trivial: over 450,000 brownfield sites were estimated to exist in the United States[2]. However, phytoremediation has not yet been widely used to clean up such sites, largely due to slow results and technical barriers.

Cadmium (Cd), a heavy metal, is frequently found in brownfields, particularly in runoff from abandoned zinc mining operations or smelting facilities, and has known health risks (Bertin and Averbeck 2006). When present at elevated concentrations in the soil, Cd requires cleanup, and for a plant to be used for such cleanup, it must be able to: (1) grow well in Cd contaminated soil, (2) accumulate high levels of Cd, and (3) transport a large fraction of the accumulated Cd to easily harvested, above-ground portions of the plant which can then be transported off-site (Karenlampi et al. 2000). Generally, plants have not evolved that can simultaneously perform these tasks, which has in turn frustrated efforts to implement Cd phytoremediation successfully. Trials using agronomic crops, which can produce high biomass, have suffered from poor

[1] http://epa.gov/swerosps/bf/index.html
[2] http://epa.gov/swerosps/bf/about.htm

uptake or poor translocation, while trials using hyperaccumulators (plants that have evolved to naturally accumulate and tolerate high concentrations of metals) suffer from low biomass, which limits total extraction. Efforts to overcome these barriers have hampered due to a limited understanding of how plants interact with metals, and critical components of plant heavy metal trafficking pathways are poorly understood. Furthermore, modeling efforts of uptake processes and responses have been limited, which also hinders development of reliable remediation systems. Thus, the focus of this research study is the measurement and modeling of Cd uptake and downstream effects on the xylem. In particular, there are some key questions: (1) what are the dynamics of root uptake kinetics and (2) what are the mechanisms plants use to transport heavy metals in the xylem to above ground tissues?

The work presented in this dissertation was conducted in an attempt to partially address the incomplete knowledge in these areas and ultimately develop better models of plant interactions with Cd. To answer the above questions, a flowing culture system was designed with the dual goals of: (1) capturing the temporal behavior of Cd uptake and modeling Cd uptake kinetics in *Brassica napus*, an agronomic plant that is capable of accumulating moderate amounts of metals, and (2) providing a platform through which xylem sap thiol contents could be analyzed in response to Cd exposure. Flowing culture systems offer some unique advantages when used for kinetic studies in that they avoid the need to characterize complex interactions between roots and a soil matrix. Such a system offers good insight to plant uptake kinetics in solution, but possibly limited insight into phytoremediation systems. Despite these limitations they have been used in understanding plant interactions with nutrients (Wild et al. 1987), and in this case, a toxic heavy metal. Furthermore, flowing culture systems have been capable of deriving whole plant uptake kinetics (Wild et al. 1987; Bloom 1989; Raman et al. 1995b), and can allow exploration of kinetic questions that other methods

cannot capture. However, initial Cd depletion profile results from using a low volume, high root mass configuration as recommended by Raman (1994) to maximize the signal-to-noise ratio found that Cd exhibited complex interactions with roots of *B. napus*. These interactions created difficulty in interpreting the depletion profile and assigning a mechanistic cause. Therefore, additional research was conducted to address the following questions:

1. What is the nature of Cd interactions with plant roots? Specifically, what is the contribution of nonspecific Cd binding to the cell wall?
2. What is the temporal behavior of Cd binding to the cell wall?
3. Are large-scale whole-plant flowing culture systems capable of resolving uptake kinetics of high affinity (low K_m) transport systems in the presence of noise generated from the cell wall binding signal?
4. What is the relationship between Cd uptake, time, and xylem sap thiols? What types of thiols are present and what is their response to Cd?

To address questions 1 and 2, a batch-based procedure was designed to understand how Cd interacted with dead roots, and how this interaction impacted flowing culture systems that rely on nutrient solution depletion in order to calculate plant uptake rates. A modeling framework based on the law of mass action, with the inherent assumptions of limited root binding sites and that binding would eventually attain equilibrium, resulted in derivation of a mathematical model that was capable of describing the temporal behavior of Cd binding to dead roots. This binding had not been previously modeled within the context of flowing culture systems and the implications for depletion measurements. Finally, to address questions 3 and 4, the flowing culture system was reconfigured and investigations were initiated into the

xylem sap thiol responses of *B. napus* to varying levels of Cd stress and time. The xylem sap thiol results revealed temporal and concentration-dependent responses to Cd and provided evidence for mechanisms plants may use in the long distance transport of heavy metals. Furthermore, the reconfigured flowing culture system revealed that it was possible to identify and isolate the noise caused by Cd binding to the cell wall, which was crucial in assigning a likely mechanistic explanation for the observed data.

2. LITERATURE REVIEW

2.1. Cadmium in the environment

Cadmium (Cd) is a transition metal and a Group II member of the periodic table. Termed a heavy metal because its density is in excess of 5 g/cm^3 (di Toppi and Gabbrielli 1999; Schutzendubel and Polle 2002), Cd was first discovered in 1817 by the German chemist F. Stronmeyer and is a known component of various naturally occurring minerals (Thornton 1986). Although present in many forms, Cd is usually found in zinc (Zn) or Zn-containing ores, and in generally small amounts in water, air, soil, and living tissues (Thornton 1986). Volcanic emissions represent the largest natural source of Cd in the atmosphere with other processes such as forest fires, sea sprays, and wind-blown dusts contributing minor amounts as well (Hutton 1983; Williams and Harrison 1986). Many natural sources and processes contribute complexity to the overall Cd cycle, and if left to their own devices, they would likely pose few problems for humans. However, anthropogenic activity has resulted in significant alterations in the Cd cycle and its subsequent distribution, which has in turn elevated concern about Cd pollution and health effects.

Anthropogenic Cd release is the result of many different activities and is significant when compared to natural Cd levels. Globally, anthropogenic sources were reported to result in about an order of magnitude increase over natural background Cd levels in the atmosphere, from 830 to 7,300 metric tons (Williams and Harrison 1986). Hutton (1983) inventoried and tabulated the annual input of Cd into various environmental compartments (air, land, and water) of the former European Community (Table 2.1). Nonferrous mines, particularly ones involving Zn or a Zn-containing ore, released Cd into all of the environmental compartments. Cd entered

Table 2.1. Annual anthropogenic Cd inputs (in metric tons/year) in the former European Community[1]

Source	Air	Land	Water
Nonferrous metal production			
Zinc and cadmium	20	200	50
Copper	6	15	--
Lead	7	40	20
Production of Cd containing materials	3	90	108
Iron and steel production	34	349	--
Fuel combustion			
Coal and lignite	8	390	--
Oil and gas	0.5	14.5	--
Waste disposal	31	1434	--
Sewage sludge disposal	2	130	33
Phosphate fertilizers	--	346	62
TOTALS	111.5	3008.5	273

[1]Data is from Hutton (1983).

the air through the high temperatures reached in smelting operations as well as wind dispersal of soil heap material; land and water Cd contamination from mining was the result of liquid wastes, leach residues, and smelter slag. Other processes, such as electroplating, pigment production, liquid and solid stabilizer manufacture, and Ni-Cd batteries contributed significantly to environmental Cd release as well, generally through landfilling and water disposal routes. Fossil fuel burning, mostly in the form of coal, contributed additional land and airborne Cd. Sewage sludge disposal and phosphate fertilizer manufacture and application represented further environmental Cd challenges. Notably, the survey showed that some releases of Cd represent particularly challenging problems: industrialized society requires enormous amounts of energy, generates large amounts of sewage sludge, and needs high-input farming to feed itself.

In examining the impact of sludge application to agricultural land, Davis (1986) noted that UK and US sludge was found to have highly variable Cd contents, with UK sludge averaging 29 mg Cd/kg dry solids. Comparatively, soils were suggested to have natural background Cd levels of 0.1-1.0 mg Cd/kg-soil (Davis 1986). Furthermore, this author calculated that UK regulatory limits would be met if typical sludge (20 mg Cd/kg dry solids) was applied to land over 30 years. Scientific uncertainty surrounding the long term fate of heavy metals added to agricultural land was also noted, and thus the wisdom of adding metal-laden amendments to such land seems questionable. From Table 2.1, a total of approximately 112, 3009, and 273 tons of Cd were added to the air, land, and water of the former European Community in a single year. More recently, a search of the U.S. EPA Toxics Release Inventory data revealed that 10.2, 5973, and 1.26 metric tons of Cd and Cd compounds were released to the air, land, and water of the U.S. in 2001. Due to environmental and health considerations, though, apparent consumption trends in the U.S. have shown an

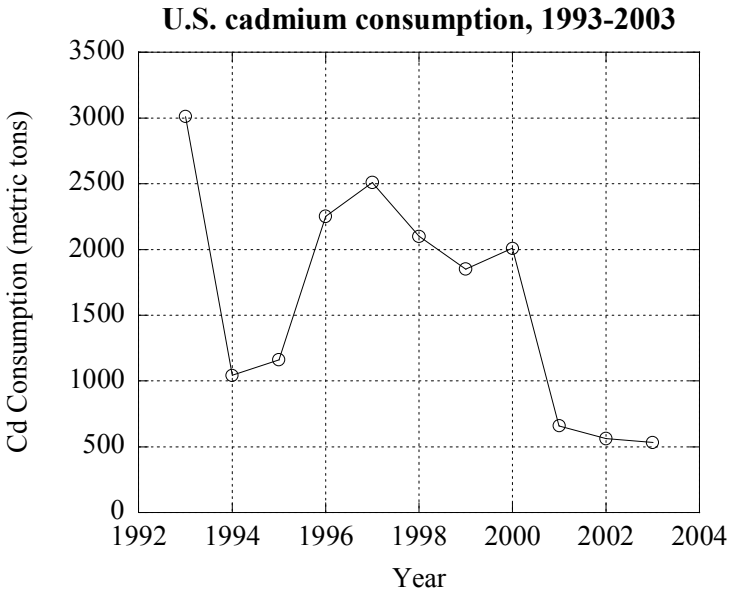

Figure 2.1. Apparent cadmium consumption in the U.S., 1993-2003. Data is from the USGS Mineral Commodity Summaries.

overall decline between 1993-2003 (Figure 2.1), which suggests that environmental Cd loading may also be declining as well.

With many different processes resulting in overall environmental Cd release, human health effects have become important and widely studied. Elevated Cd exposure (either chronic or acute) generally results in a range of undesirable health effects in humans. This exposure can come from many sources, including contaminated water, food, or air. Lifestyle choices, such as smoking, also impact Cd body burden; smokers were found to have approximately double the Cd body content of non-smokers (Ellis et al. 1979). Perhaps the best known poisoning incident was the occurrence of itai-itai ('ouch-ouch') disease along the Jinzu River in the Toyama Prefecture of Japan that was linked to elevated Cd exposure from an upstream zinc-lead mining operation (Korte 1983). Pain in the extremities, walking difficulties, bone fractures, and spinal defects were all major symptoms of the disease. Renal tubular dysfunction, osteomalacia, anemia, proteinuria, aminoaciduria, glucosuria, and possibly hypertension are all common effects of Cd exposure. Effects of acute exposure are usually delayed by hours or even days, but have included severe respiratory distress, nausea, vomiting, dizziness, shortness of breath, and other undesirable conditions (Bernard and Lauwerys 1986; Shukla and Singhal 1984; Hallenbeck 1986). Thus, given the various damaging health effects caused by Cd and the multiple anthropogenic activities that lead to Cd release in the environment, a clear need exists for technologies that either do not require Cd or are able to prevent its unsafe release. Likewise, a need exists for technologies that can effectively and economically clean up areas that have been contaminated with Cd.

2.2. Phytoremediation

2.2.1. Definition, types, alternatives, and costs

The term 'phytoremediation' first appeared in the literature around 1993 and is defined as a process whereby plants are used to clean up the environment (Salt et al. 1995a). Phytoremediation approaches currently fall into six main areas as originally defined by Salt (1998):

- Rhizofiltration—using plants to remove pollutants from aqueous waste streams;
- Phytodegradation—using plants and root-associated microorganisms to degrade organics;
- Phytoextraction—using plants to accumulate and remove pollutants by translocating them to above-ground portions of the plant;
- Phytovolatilization—using plants to volatize pollutants;
- Use of plants to remove airborne pollutants; and
- Phytostabilization—using plants to reduce pollutant bioavailability.

This technology is applicable to both heavy metal and organic pollution, although a suggestion has been made that plants are less suitable than microbes for remediation of organic contaminants due to incomplete mineralization and possibly toxic metabolic byproducts (Salt et al. 1998). However, reviews (Macek et al. 2000; Singh and Jain 2003) have suggested that various classes and types of chemicals such as PCBs, TCE, TNT, petroleum hydrocarbons, RDX, PCPs, and organophosphate insecticides were amenable to phytoremediation approaches. Important considerations for organics were stated to include the octanol-water partition coefficient (K_{ow}) whereby moderately hydrophobic compounds are more likely to enter the plant and undergo

degradation; lower hydrophobicity values present problems in terms of insufficient root sorption and difficulties crossing the cell membrane, while higher degrees of hydrophobicity make translocation within the plant difficult (Dietz and Schnoor 2001). The authors noted, however, that exceptions to this relationship exist. Uptake of organics was found to be a function of a plant's uptake efficiency (termed the transpiration stream concentration factor, TSCF), transpiration rate, and chemical concentration in the soil water (Dietz and Schnoor 2001). Concerns about incomplete transformation and toxic intermediates were again noted (Macek et al. 2000), but plant remediation and engineering efforts have suggested that phytoremediation of at least some classes of organic pollutants is feasible (Gao et al. 2000b; Gao et al. 2000a; Macek et al. 2000; Singh and Jain 2003).

In contrast to organics, plants have represented the only really viable option for biological remediation of heavy metals (Raskin 1996). Such metals cannot undergo further chemical degradation to reduce their toxicity per se, although some can undergo a change in oxidation state. Perhaps the best known plant example of this phenomenon is iron (Fe) uptake in plants, where Strategy I plants, upon obtaining Fe(III) from the soil, have been shown to reduce it to Fe(II) using a membrane-bound reductase before transporting it into the cell (Marschner and Romheld 1994). For the purposes of mercury (Hg) phytoremediation, genetic engineering of *Arabidopsis* and yellow poplar yielded plants that were capable of taking toxic Hg^{2+} and reducing it to Hg^{0} which was subsequently volatilized (Rugh et al. 1996; Rugh et al. 1998). Further efforts (Bizily et al. 1999; Bizily et al. 2000) yielded plants that reduced highly toxic methylmercury to Hg^{2+} and subsequently Hg^{0}. Selenium (Se), although not a heavy metal, was another element that was reduced and volatilized by plants in the form of dimethylselenide (de Souza et al. 1998; Pilon-Smits et al. 1998). More recent genetic engineering studies showed some promise in enhancing the ability of plant-based

remediation of this element (Pilon et al. 2003; Van Huysen et al. 2003). Evidence has also been presented for reduction, but not volatilization, of arsenic (As) in at least one species; in *Brassica juncea* roots, arsenate, As(V), was reduced to As(III) (Pickering et al. 2000). However, most heavy metals, including Cd, cannot be volatilized, easily reduced, or changed to a less toxic form, and thus the only option for remediation is physical separation and removal from their environment.

This need for separation and removal, rather than in situ detoxification, has driven up the cost of remediating soil or water contaminated with heavy metals. In a recent review, various technologies for cleaning up metal laden soil and groundwater were discussed (Mulligan et al. 2001). These methods included the following:

- Solidification/stabilization—solidification physically encapsulates the contaminants; stabilization includes chemical reactions that reduce metal mobility;
- Mechanical separation—larger, cleaner particles are separated from smaller ones that are more polluted;
- Pyrometallurgical separation—furnaces are used to volatilize metals;
- Vitrification—where electrodes and thermal energy are used to vitrify the mass containing a contaminant
- Chemical treatment with oxidative or reductive mechanisms;
- Permeable treatment walls—these walls contain a reactive substance;
- Electrokinetics—uses a low intensity electric current to move and concentrate ions for easier treatment through chemical precipitation, exposure to ion exchange resins, etc.;
- Biological processes, including bioleaching, biosorption, bioprecipitation, (all using bacteria) and phytoremediation;
- Soil flushing—uses an extracting compound and performed in situ; and

- Soil washing—uses stronger, more concentrated chemicals performed ex situ

The cost of these different technologies varies widely; Mulligan et al. (2001) estimated the cost at $10-$1000 per ton for the non-biologically based methods. Furthermore, several of these methods represent only partial remediation: solidification/stabilization and vitrification strategies simply seek to limit pollutant movement and precipitation strategies change only the chemical behavior of the metal while leaving it in the soil or water unless further removal steps are taken. However, one should note that these technologies do have important applications, particularly in areas where metal concentrations are too high or the environment too inhospitable to support plant growth. Areas where contamination is deep underground or underwater, or where quick cleanup is mandated also represent sites where current conventional technologies have an advantage over phytoremediation.

With this in mind, phytoremediation is best suited for sites with moderate levels of pollution, relatively shallow (< 7 m) contamination depths, and generous timetables for cleanup. In areas that meet these criteria, plant-based technologies potentially have a significant economic advantage over other treatment options. However, widely varying cost estimates were found in the literature, which made it difficult to assign a precise value to what the purported cost advantage might be. Some authors have attempted to estimate phytoremediation costs and then compare them to estimated costs using conventional technology; these estimates are presented in Table 2.2. Site-specific costs and lack of economic data on this technology were further noted as complicating factors in obtaining good cost estimates (Lasat 2002). Salt et al. (1995) provided a market estimate of $400 billion for hazardous material cleanup in the U. S. using conventional (non-biologic) methods and stated that phytoextraction of an acre (50 cm depth) will cost between $60,000 - $100,000 compared to $400,000 per acre or greater using other methods. Other authors have

Table 2.2. Comparison of cost estimates for phytoremediation versus conventional technology

Conventional technology cost	Phytoremediation cost	Author(s)
\$10 – \$1,000/ton (\$45,000 – \$4,500,000/acre[+])	\$50,000 – \$200,000/acre	Mulligan (2001)
≥ \$400,000/acre	\$60,000 – \$100,000/acre	Salt et al. (1995)
\$1,000,000/acre	Not provided	Salt (1998)
≥ \$250,000/acre	Factor of 10^2 – 10^4 "less"	Cunningham et al. (1996)
\$3,000,000/acre	\$101,000/acre	McGrath et al. (2001)
≥ 2 x phytoremediation cost	\$25 – \$100/ton (\$112,000 – \$450,000/acre[+])	Macek et al. (2000)
\$243,000 – \$1,214,000/acre	Not provided	Moffat et al. (1995)
\$85,600[*]/acre (capping cost)	\$14,300[*]/acre	Robinson et al. (2003a)

[*]Monetary figures were converted from New Zealand Dollars using the 4/14/04 exchange rate

[+]Assuming 1 acre to a depth of 3 feet weighs 4500 tons, from Cunningham et al. (1996)

suggested different phytoextraction costs, with a low estimate of just over $14,000/acre and a high estimate of $450,000/acre. Robinson et al. (2003) recently provided their phytoremediation cost estimate based site specific conditions. Using popular trees, a boron-laden sawdust pile (3.5 ha) was undergoing remediation in order to reduce boron (B) loading to a nearby stream. These authors stated that site assessment including a plant trial and chemical analysis was responsible for half of the cost estimate, which included a 5 year management plan (Robinson et al. 2003a). Site capping was stated to be the conventional treatment alternative, at a cost of over $85,000/acre. Clearly, the assumptions made about depth and degree of contamination were important; while all authors stated that phytoremediation would cost less than other alternatives, quantifying the amount of money saved was much more difficult. In some cases, the exact alternatives that phytoremediation was being compared to were not clear, and given that there are many types of conventional methods available with varying costs, specifying the technology that plant-based systems are compared to would be helpful. Intuitively, one may expect that growing plants to clean up a heavy-metal contaminated field will cost less than more traditional engineering approaches, but since phytoremediation is a young technology with limited data, this expectation hasn't yet been rigorously tested.

2.2.2. Phytoextraction and rhizofiltration

Phytoextraction, as defined earlier, uses plants to extract and translocate metals to above-ground plant biomass. Therefore, characteristics an ideal plant should possess for this technology have been suggested to include the following: accumulation of the target metal and translocation of it to above ground portions of the plant, tolerance of high metal concentrations, quick growth, production of high

biomass, and finally, easy harvestability (Karenlampi et al. 2000). Unfortunately, such a plant does not yet exist for most pollutants. Generally, investigators used two types of plants: agronomic plants with high biomass such as *Brassica juncea*, *Zea mays*, or *N. tabacum*, or plants that naturally accumulate exceptionally high levels of metals in their tissues, called hyperaccumulators. Salt (1998) identified two basic metal removal strategies: (1) induced phytoextraction for ones that used an amendment to enhance plant uptake, and (2) continuous phytoextraction for ones that relied on natural plant processes for metal accumulation. The term "hyperaccumulator" first appeared in a 1977 study describing nickel (Ni) accumulation in plants (Brooks et al. 1977). Since then, the term has expanded to include plants that concentrate abnormally high levels of any given metal. In other words, the definition is relative for a given metal based on abundance data for normal plants, and thus the threshold has been set at 10,000 mg/kg for Zn and 1,000 mg/kg for cobalt (Co), copper (Cu), and Ni (Baker and Brooks 1989). For Cd, the value has been set at 100 mg/kg, although these numbers were suggested to be more like guidelines rather than an absolute level requirement before designation of hyperaccumulation status (Baker and Whiting 2002).

In terms of species number, the group of hyperaccumulating plants continues to grow as new discoveries are made. Baker and Brooks (1989) reported on about 225 known species of hyperaccumulators (145 of them for Ni) in geographically disparate areas in both temperate and tropical climates. Another report suggested a total of about 700 species in its introduction (Robinson et al. 1997), although it was unclear how the authors arrived at this number. More recently, the number of such plants was placed at around 400, with the majority of these plants (317 or more) classified as Ni hyperaccumulators (Assuncao et al. 2003b). However, in addition to Ni, the total list recently included plants that accumulate Zn, Cd, Se, Pb, Cu, As, Co and Mn

(Assuncao et al. 2003b; Cobbett 2003). Hyperaccumulating plants contain varying levels of metal, dependent on the plant, metal, soil type, and growth conditions. In terms of a Cd phytoextraction setting, slow growth and/or low biomass production have been nearly universally cited as the main constraint to using hyperaccumulating plants for heavy metal remediation (Kumar et al. 1995; Cunningham and Ow 1996; Ebbs and Kochian 1997; Salt et al. 1998; Karenlampi et al. 2000; Kayser et al. 2000; Xiong and Feng 2001). However, the discovery of a large-biomass, fast growing Ni hyperaccumulator in southern Africa called *Berkheya coddii* (Robinson et al. 1997) indicated this is not a universal constraint. Robinson et al. (1997) reported biomass production of about 22 t/ha for this plant with an average Ni content of 7880 mg/kg under fertilized conditions, which put this hyperaccumulator in roughly the same productivity range as *Zea mays* (30 t/ha, fertilized). This productivity was significantly greater than that of *Thlaspi caerulescens* (5.1 t/ha, fertilized), a frequently studied metal hyperaccumulator. Interestingly, when shoot biomass was excised from *B. coddii*, the new shoot tissue quickly grew back and contained a higher Ni concentration than the excised material (Robinson et al. 1997), which suggested a possible way to manipulate some plants into accumulating even more metal. Importantly, this work demonstrated that hyperaccumulating plants were not limited to small, slow growing types, and hence efforts directed towards finding additional high-biomass accumulators should continue. Furthermore, additional research into this species may provide ways to enhance biomass production of known hyperaccumulators.

Table 2.3. Results from studies using hyperaccumulators

Species	Shoot Cd (mg/kg-DW)	Biomass	Time	Total Cd extraction	Total soil Cd (mg/kg DW)	Author(s)
T. caerulescens	37-1740	0.2-4.25 g/plant	38 d.	29-1032 μg/plant	38-1020; Zn present	Brown et al. (1994)
A. halleri	20-504	.31-1.92 g/plant	61 d.	12.4-304 μg/plant	0.8-50; Zn, Pb, Cu present	Dahmani-Muller et al. (2001)
A. halleri	281 (leaves) 50 (stem)	Not provided	Unknown	Not provided	23-197; Zn, Pb, Cu present	Dahmani-Muller et al. (2000)
T. caerulescens	1618 (1 yr. plot) 527 (2 yr. plot)	2.6 t/ha	2 yr.	4.2 kg/ha	1-360; Zn, Pb present	Robinson et al. (1998)
T. caerulescens Lettuce	1-28 1-29	5.53-7.6 g FW Not provided	2 yr.	1.1 μg/plant	1.01-5.46; Zn also present	Brown et al. (1995)
T. caerulescens	50-275	0.6-2.1 t/ha	Varied	128, 539 g/ha (transpl) 85, 184 g/ha (sown)	2.5-2.8; Zn, Cu present	Hammer and Keller (2003)

2.2.3. Hyperaccumulators and phytoextraction

Despite these so-called biomass and growth limitations, some investigators have used these metal-rich plants for studying their utility for Cd phytoextraction on polluted soils. The results of several of these studies are summarized in Table 2.3. Attempts have been made to standardize these results, when possible, to facilitate comparisons across studies. In general, investigators either attempted to view growth and metal accumulation in a field setting, or they tried to vary soil parameters in more controlled settings to enhance metal uptake. Brown et al. (1994) compared three species and varied the pH, using reduced sulfur or calcium hydroxide, of Cd and Zn laden soils collected around a Zn smelter. Lower pH values were generally shown to enhance shoot metal concentrations of all three species, and *T. caerulescens* extracted more metal than *Silene vulgaris* and Rutgers tomato (*Lycopersicon esculentum* L.), but 15 years would still be required to clean the site (Brown et al. 1994).

The field and pot studies on *Arabidopsis halleri* (Dahmani-Muller et al. 2000; Dahmani-Muller et al. 2001) demonstrated the ability of this plant to achieve high Cd concentrations, but the field study did not provide enough information to calculate total metal extraction. In studying wild populations of *T. caerulescens* growing in the metal-contaminated Les Malines region of southern France (163 mg/kg soil Cd content), Robinson et al. (1998) found elevated and time dependent plant Cd levels. Two year old field plants had significantly lower Cd levels than one-year old field plants, and about 18 years would be required for total site cleanup (Robinson et al. 1998). However, these authors suggested field biomass production could be doubled by fertilization, which may increase overall extraction and thus reduce this time. Another study (Brown et al. 1995) compared extraction capabilities of *T. caerulescens*, lettuce, and *S. vulgaris* on sludge-amended field plots using two soil pH

levels. Here, sludge source showed a statistically significant effect and the lower pH treatments resulted in higher plant Cd levels at the two highest Cd loading rates. Interestingly, *T. caerulescens* failed to extract more Cd than lettuce.

More recently, *T. caerulescens* was grown on two field sites (Dornach, with brass smelter contamination and Caslano, with sewage sludge contamination) in Switzerland over a period of two years (Hammer and Keller 2003). They examined both plant metal levels and the effects of sowing versus transplanting the hyperaccumulator. Three successive crops were transplanted the first year and one "long crop" was field sown the second year. Even though the two sites had similar Cd levels, site specific effects on plant metal yield were apparent as Caslano plants consistently had higher Cd concentrations, regardless of growing scheme. Maximum plant Cd yield was about 270 mg/kg DM (Caslano), but metal yield decreased by over a factor of two at both sites by the third transplant. The continuous crop showed relatively consistent metal levels throughout the trial. Overall, no statistically significant difference was found between the two growing strategies over three harvests (Hammer and Keller 2003). Biomass yields were inconsistent at both sites, which possibly suggested non-optimized agronomic practices.

Thus, the development of Cd phytoremediation using natural hyperaccumulators is in its early stages, and several problems are worth noting. One major problem is the shortage of full-scale, long term (> 2 years) field trials that would attempt to examine the performance of such plants in the field (Baker and Whiting 2002). Long term field trials using these plants are critical in evaluating their performance and the problems that would be encountered in the field. They would greatly assist development of the engineering and science that is needed for implementation of phytoextraction technology, and help close the gap between "science and practicality" that Baker and Whiting (2002) warned about. Such trials

would help optimize hyperaccumulator agronomic practices, provide field validation for phytoremediation technology, and establish common (and perhaps unexpected) problems encountered at field sites as well as mitigation strategies. For instance, biomass production may significantly differ between controlled and field conditions. Hammer and Keller (2003a) experienced this problem—at best, they were able to achieve 1.8 t/ha under fertilized conditions (with most of their trials significantly lower), which is far below the yield suggested by Robinson et al. (1998) of 2.6 t/ha unfertilized, and 5.2 t/ha fertilized. No standard agronomic practices yet exist for hyperaccumulators, and none ever may if a reliable way to enhance biomass production isn't found. Another problem is the lack of agronomic machinery for hyperaccumulators, and if existing equipment cannot be easily modified and highly specialized machinery must be developed, costs for implementing this technology will likely increase.

Also, some hyperaccumulators do not uniformly display metal accumulation and resistance properties; for instance, significant variation has been observed between populations or ecotypes of *S. vulgaris*, *A. halleri*, and *T. caerulescens* (Ernst et al. 1990; Bert et al. 2002; Assuncao et al. 2003a). Specific cultivars, then, need to be developed and seed supplies enhanced so that availability is less of a problem. Another problem is that knowledge about plant heavy metal biochemistry is far from complete, particularly in areas involving transport and sequestration. Hyperaccumulators and their unique capabilities are perhaps even more enigmatic, although the sophisticated research tools available in this era of "omics" may help to elucidate more aspects of plant heavy metal trafficking in the near future. In short, these issues have probably all contributed to the rather variable results seen in field studies using known hyperaccumulators to remediate Cd thus far, and clearly some

important issues must be addressed for hyperaccumulator use in phytoextraction to proceed.

2.2.4. High-biomass plants and induced phytoextraction

Some of the aforementioned problems with hyperaccumulators have led investigators to use higher biomass producing plants—such as agronomic crops or trees—in phytoextraction research. These species are generally better characterized with several cultivars and readily available seed supplies. The disadvantage, however, of using such species is that they usually do not accumulate high levels of metals in their above-ground parts, which reduces their extractive capabilities and overall utility. Furthermore, these plants may not be metal tolerant, thus hampering germination and growth at a contaminated site. In order to partially remedy these problems, the use of chelators and other soil amendments was suggested by Salt et al. (1995) to help non-hyperaccumulating species acquire more metals from the soil. Thus investigators, whether conducting field or laboratory studies, have frequently used chemical amendments to enhance growth, metal accumulation, or both. Various studies have also attempted to identify suitable high-biomass species for phytoextraction (Kumar et al. 1995; Huang and Cunningham 1996; Ebbs and Kochian 1997; Ebbs et al. 1997; Ebbs and Kochian 1998; Keller et al. 2003). Results from several of these studies have been summarized in Table 2.4. Again, attempts were made to place results on an equivalent basis in order to facilitate cross-study comparisons.

Generally, only a few of the studies achieved hyperaccumulator-like concentrations in above ground plant biomass. In one such study, *Brassica juncea* was grown in an artificially contaminated sand/perlite mixture with fertilization and was able to reach Cd hyperaccumulator status (Kumar et al. 1995). Blaylock et al.

Table 2.4. Phytoextraction using high biomass plants

Species	Shoot Cd^{2+} (mg/kg-DW)	Biomass	Time	Total Cd^{2+} extraction	Total soil Cd^{2+} (mg/kg DW)	Author(s)
B. juncea	104	Not provided	14 d.	Unknown	2	Kumar et al. (1995)
B. juncea	220 2800 (EGTA)	2g DW/pot 1.17 g DW/pot	28 d.		100	Blaylock et al. (1997)
B. juncea	2.3	34 g DW	42 d.	73 μg/pot	40 Cu, Zn	Ebbs et al. (1997)
B. rapa	2.4	33 g DW		95 μg/pot		
B. napus	2.5	25 g DW		68 μg/pot		
F. rubra	0.3	24.8 g DW		8 μg/pot		
A. capillaries	1.1	14 g DW		30 μg/pot		
Z. mays (EDTA)	0.81-4.13 (UK) 3.8-40.2 (FR)	8.5- 15.7 g DW 0.44-12.5 g DW	~100 d.	9.6-35.1 μg/plant (UK) 17.7-61.3 μg/plant (FR)	42 (UK) Cu, Pb, Zn 19 (FR) Cu, Pb, Zn	Lombi et al. (2001)
B. rapa (EDTA)	7	Not provided	28 d.	Unknown	5.5; Pb, Zn present	Grcman (2001)
H. annuus	110 (EDTA)* 95 (HEDTA)* 48 (control)*	1.5 g DW 1 g DW 4.2 g DW	28 d.	22.2 μg/plant 12.2 μg/plant 66.7 μg/plant	30 (each of Cd, Cr, Ni)	Chen and Cutright (2001)
B. napus	25.33 (leaves) 11.94 (stems)	0.409 g DW 0.772 g DW	Unknown	14 μg	59.68	Rossi et al. (2002)
S. viminalis	6.3-1.9 (S_8, Dorn.) 3.6-2.4 (Caslano)	25-35 t/ha	5 yr. 2 yr.	170 g/ha 47 g/ha	2.8	Hammer et al. (2003)
S. viminalis	0.6-4.1 (stems) 1-7.3 (leaves)	2.1-8.7 t/ha-yr 2.0-7.8 t/ha	3 yr.	2.6-16.5 g/ha-yr.	0.45-0.1	Klang-Westin and Eriksson (2003)
T. aestivum L.	55.4-111.2 (EGTA+ Cd) 3.2-36.7 (controls)	.0527-.0797 g DW .0625-.0827 g DW	42 d.	0.13-5.9 μg/plant	0-1000	Begonia et al. (2003)

**Concentration data from this study was from Cd-only pans, but biomass and total extraction data was reported from pans containing 30 mg/kg soil DW mixed metals.*

(1997) conducted a pot experiment where soil pH levels were varied and different chelators were used to examine heavy metal uptake in *B. juncea* grown on soil amended with $CdCO_3$. Although plants grown in the absence of chelators were able to achieve high shoot metal concentrations, 10 mmol/kg soil EGTA raised these levels even further (Blaylock et al. 1997). Shoot Cd levels were sharply reduced, though, in pots with multiple metals and EDTA, which suggested that soil and competition effects as well as metal:chelator equilibria were important factors to consider when selecting a remediation strategy. Chen and Cutright (2001) grew *Helianthus annuus* in pans on soil artificially contaminated with Cd, Cr, and Ni and compared the effects of 1 g/kg soil (~2.6 mmol/kg soil) or 0.5 g/kg soil (~1.3 mmol/kg soil) of HEDTA or EDTA. Although both chelators raised shoot Cd levels to around hyperaccumulator thresholds, the plants experienced severe phytotoxicity, exhibited sharply reduced biomass, and extracted less total metal than control (no chelate) plants (Chen and Cutright 2001). Finally, *T. aestivum*, or wheat, was pot grown on artificially contaminated soil and yielded shoot Cd concentrations of over 100 mg/kg on soil containing 1000 mg Cd/kg soil and EGTA at 5.0 mmol/kg soil (Begonia et al. 2003). However, the Cd levels used may not be observed frequently in the field or be environmentally relevant and the EGTA application rate may be too high for practical field use. Importantly, all of these studies that reported high plant metal concentrations used artificially contaminated growth media, which does not mimic *in situ* behavior of metals at actual field sites (Robinson et al. 1998). Therefore, the results of these studies may be difficult to replicate under field conditions.

Other investigations that used either media obtained from industrially or sludge-contaminated sites have yielded mixed results. After screening 30 different species grown in hydroponics for metal accumulation, Ebbs et al. (1997) grew three *Brassica* and two grass species in pots containing soil that was contaminated with Cd,

Cu, and Zn from an upstream mine and compared their performance to the hyperaccumulator *T. caerulescens*. Various amendments, including fertilizer, compost, and sewage sludge were added to the pots to see if they enhanced shoot metal levels. Only Gro-Power (one of the amendments) exerted a statistically significant effect on Cd accumulation, and this effect was only observed in *B. juncea*. However, as indicated in Table 4, overall plant Cd levels were relatively low and unfortunately not enough data was provided to allow for a calculation of per-plant extraction. Here, the *Brassica* species (due to their 10-fold higher biomass) were just as effective as the hyperaccumulator in total Cd removal (Ebbs et al. 1997). Lombi et al. (2001) conducted a pot study and used two contaminated soils and compared metal extraction of *T. caerulescens* and *Zea mays*. Three successive croppings were conducted over a 391 day period, and EDTA was used to enhance *Zea mays* metal accumulation. In this case, corn accumulated significantly more metals in the roots than shoots, and was overall a poor metal extractor due to poor uptake, root to shoot metal translocation and stunted growth, even though EDTA did increase metal content (Lombi et al. 2001a). Notably, an attempt at using *B. juncea* failed due to severe phytotoxicity on this soil even in the absence of EDTA, which suggested the need for metal tolerant varieties and that currently available agronomic crops may not be suitable for some sites. Grcman et al. (2001) used a single 10 mmol/kg soil EDTA application to soil columns from an industrially contaminated site to enhance leaf Cd, Zn, and Pb levels in *Brassica rapa* when compared to controls and lower or sequential chelate additions. However, shoot metal levels were still rather low, and the most effective treatment resulted in lower plant biomass and senescence (Grcman et al. 2001).

Another *Brassica* species, *Brassica napus*, accumulated moderate Cd levels in the stems and leaves, but was grown on soil mixed with municipal solid waste that

was artificially contaminated with Cd (Rossi et al. 2002). Municipal solid waste did not increase plant biomass (Rossi et al. 2002) and *B. napus* clearly did not achieve hyperaccumulator values, although more Cd was found in shoot biomass than in the roots. More recently, *Salix viminalis* (Hammer et al. 2003; Klang-Westin and Eriksson 2003) was studied for potential Cd remediation. Hammer et al. (2003) grew *S. viminalis*, or willow, on Cd, Zn, and Cu smelter or sludge contaminated field plots for either two or five years. Plant Cd concentrations were low, decreased over time, and single sulfur or Fe fertilizer amendments appeared to have temporary effects that failed to yield statistically significant improvements in long term metal concentration or total extraction. Also, despite high biomass production of 25-35 t/ha, *Salix* was less efficient at metal removal than *T. caerulescens* because its roots grew beneath the zone of contamination, which reduced its extraction efficiency (Hammer et al. 2003). Klang-Westin and Eriksson (2003) found similarly low Cd levels in *Salix*, although growth was on sludge or fertilizer-amended agricultural land with very low (< 0.5 mg Cd/kg soil) existing Cd levels.

Thus, the use of high biomass plants and EDTA or similar chelators for phytoextraction of Cd had various outcomes. An important point regarding these types of synthetic chelators must also be considered: several authors noted the risk of EDTA or similar chelators forming highly soluble complexes that may percolate through the soil profile and leach into groundwater supplies (Stanhope et al. 2000; Lombi et al. 2001a; McGrath et al. 2001; Lasat 2002). Grcman et al. (2001) found substantial amounts of Pb, Cd, and EDTA in soil column leachates. Furthermore, phospholipid and diglyceride fatty acids analysis (PFLA and DGFA) suggested EDTA induced stress on soil microbiota, particularly fungi, and the authors suggested the need for chelate risk assessments for contaminated sites. In outdoor pot and field lysimeter phytoextraction experiments on industrially contaminated soils, leaching of

metals was recorded even at EDTA additions as low as 0.25 g/kg soil (Wenzel et al. 2003). Additionally, the authors noted elevated metal levels in soil leachate many months after EDTA addition, which suggested extended persistence and mobility of EDTA-metal complexes. Wenzel et al. (2003) concluded that development of continuous phytoextraction technologies was preferable to chelate-induced methods.

In order to partially alleviate these concerns, some authors have searched for alternative chelators or better management strategies for the application of metal mobilizing amendments to contaminated sites. One study (Kayser et al. 2000) examined the impact of the chelator nitrilotriacetate (NTA) and soil acidifying elemental sulfur (S_8) on enhancing phytoextraction. The authors grew five crop species and two hyperaccumulating species on Zn-smelter contaminated field plots (elevated Cd, Zn, and Cu) for two years, adding NTA or S_8 during the second year. NTA strongly increased $NaNO_3$-extractable metal concentrations after application, but yielded modest, if any, increases in plant Cd concentration (Kayser et al. 2000), with generally poor uptake observed in all species. This study was a good example of the difficulties encountered when working with field sites, where even hyperaccumulating species can perform poorly. Recently, citrate was shown to be effective at desorbing Cd from contaminated soil (Gao et al. 2003). However, the application rate of several thousand kilograms per acre that would be required to match experimental conditions may not have been realistic or possibly too expensive for field use. Another report used citric acid (Chen et al. 2003) to desorb Cd from artificially contaminated soil, but application rates and subsequent cost may have again been too high for field use.

Other authors suggested the use of biodegradable biosurfactants such as surfactin and NaOH or sophorolipid and HCl (Mulligan et al. 2001) for metal removal, although their use in a phytoremediation setting has yet to be tested. In a recent review (Schmidt 2003), studies using ammonium sulfate $(NH_4)_2SO_4$, chloride salts,

and liming as amendments to assist Cd phytoextraction were discussed, although no one amendment appeared to stand out above the others, with some giving inconsistent results. Schmidt (2003) characterized the relative effects of various soil amendments for Cd accumulation as "small" for EDTA, CDTA, NTA, organic acids, ammonium sulfate, and salts, and designated sulfur as the only amendment with a "medium" effect for Cd. This is in spite of the fact that soluble Cd and other heavy metal concentrations in the soil were dramatically enhanced by more than one type of amendment, which suggested that metal solubility may not always be the limiting factor in phytoextraction. In addition to chelators or chemical soil amendments, modification of a plant's microclimate may also enhance metal accumulation. *Brassica pekinesis* (commonly called Chinese cabbage) exhibited approximately twice the biomass production when grown under floating row covers in the field, and as a result, extracted significantly more heavy metal (Moreno et al. 2002). While this study was conducted on normal soil with low heavy metal levels and thus caution must be exercised in extending its applicability to phytoextraction, it did suggest that specialized agronomic practices may also be important in developing effective phytoremediation schemes.

Clearly, Cd phytoextraction using high biomass plants and/or chemical amendments is not yet a mature technology given varying results reported in the literature. This variation is partially due to limited understanding of the underlying biological processes governing plant heavy metal uptake. For instance, the biological mechanisms responsible for plant chelate-metal uptake and transport are not yet well understood. However, a report that examined EDTA and Pb transport in *B. juncea* that was grown in hydroponics (Vassil et al. 1998) yielded some interesting results. The authors found evidence for Pb-EDTA transport in the xylem sap, which suggested that Pb-EDTA was transported from roots to shoot in this manner. Additionally, a

threshold EDTA level was needed in order to induce high EDTA uptake rates, which possibly indicated a breakdown of the plant mechanisms that normally governed uptake (Vassil et al. 1998). Berkelaar and Hale (2003) studied Cd and Cd-ligand uptake at low concentrations in two wheat cultivars that were also grown in hydroponics and found enhanced root Cd levels in the presence Cd + EDTA when compared to Cd-only solutions. They did not assay for xylem or plant EDTA, but did suggest Cd-EDTA uptake as a plausible mechanism. Unfortunately, mass transfer limitations may have clouded their results (Berkelaar and Hale 2003). How applicable these findings were to other EDTA-metal complexes or other chelator complexes is not clear, and it's unknown if these same mechanisms work in soil-based field settings.

2.2.5. Total metal extraction

In considering metal phytoextraction, total metal removal is an important performance parameter and must be stressed. The amount of metal a plant extracts is given by the following simple equation:

$$M_{EP} = (S_{MC}) \times (S_M) \qquad \text{[Eq. 2.1]}$$

where:

M_{EP} represents the metal extracted per plant;

S_{MC} represents the plant shoot metal concentration; and

S_M represents shoot mass on a wet or dry weight basis in kg.

Thus, at any given time, the total metal removal per unit land area is given by:

$$M_{RE} = \frac{(M_{EP}) \times (N_P)}{A_{PC}} \qquad \text{[Eq. 2.2]}$$

where:

M_{RE} is the metal removal per unit land area;

M_{EP} is defined in Eq. 2.1;

N_P represents the number of plants; and

A_{PC} represents the plant coverage area (m^2, acres, or hectares)

Importantly, M_{RE} is a function of time since shoot mass (S_M) certainly changes with time, and shoot metal concentration (S_{MC}) is time dependent as well. Additionally, the total metal removal from a site is the summation of M_{RE} over several croppings:

$$\sum_{i=1}^{n}\left(M_{RE}\right)_i \qquad \text{[Eq. 2.3]}$$

Various authors (Brown et al. 1994; Brown et al. 1995; Robinson et al. 1998; Hammer and Keller 2003) have attempted to estimate the time required to remediate the particular soil used in their study. These authors based their estimates on the assumption that M_{RE} was invariant over the course of several croppings—that is M_{RE} at $i = 1$ is the same as M_{RE} at $i = 15$ or greater. Essentially, zero order extraction kinetics were assumed. Due to the absence sufficiently long term field studies, this assumption has not been tested. However, M_{RE} could change over time, owing to changes in soil chemistry and metal bioavailability or other unknown factors, and constant uptake assumptions can lead to an underestimation of the time required to remediate a site. Also, Eq. 2.2 was based on the assumption that each plant extracts approximately the same amount of metal. Hence, great importance must be placed on development of adequate sampling protocols that yield mean extraction values which could be used with some confidence in Equation 2. Ideally, an extracting plant would have shoot metal concentrations approaching that of a hyperaccumulator and annual biomass production similar to an agronomic crop such as corn. For phytoremediation to become widely adopted and enter mainstream engineering practice, these goals are paramount.

2.3. Cadmium in plants

Due to their sessile nature, plants have been forced to evolve sophisticated cellular machinery in order to both grow and cope with environmental stresses. In this section, some aspects of plant heavy metal biochemistry are reviewed with particular emphasis placed on Cd effects and the molecular transport network. Advances over the past 10+ years have allowed for a much more detailed understanding of the effects nonessential heavy metals, such as Cd, have on plants as well as the mechanisms plants use to mitigate and avoid these effects. With genomic data widely available and expanding, a significant amount of recent research has been devoted to understanding what genes a plant uses to transport and sequester metals at the cellular level in the ultimate hopes of improving food safety, nutrition, and possible use of plants for phytoremediation. To date, several different classes of transporters have been identified, although current knowledge about structure, function, and physiological relevance is far from complete. This lack of basic knowledge has hampered efforts to rationally design an "ideal plant" for phytoremediation, although ongoing and future endeavors may solve some of these problems.

2.3.1. Plant heavy metal transporters

Contemporary research tools have elucidated much information on how plants manage metals at the cellular level. When considering Cd and the problem of environmental remediation, one must keep in mind that plants do not have transporters that are specifically designed for Cd uptake since it is not a required nutrient for normal plant growth and has no known physiological function. Instead, Cd enters the plant through what are now known to be a wide array of molecular transporters that

Table 2.5. Summary of probable heavy metal transporters and location[1,2]

Transporter class	Cellular location
ZIP (ZRT, IRT-like proteins)	Plasma membrane, plastid, other intracellular compartments
P-type ATPases (P_{1B}-ATPases)	Intracellular membranes, plasma membranes plastid, ER, Golgi, no evidence for vacuole
NRAMP	Plasma membrane, vacuole, plastid
CDF/CE (cation diffusion facilitator/cation efflux proteins)	Vacuole, intracellular membranes
Cation/H^+	Vacuolar membrane
CNGC	Plasma membrane
MATE	Plasma membrane
COPT	Plasma membrane
ABC	Vacuolar membrane
OPT	Intracellular

[1]From Clemens (2001), Hall and Williams (2003).
[2]Note that the number of classes and cellular location(s) may change as research advances and additional transporter classes may be found to have important roles in heavy metal transport.

have primary functions of acquiring physiologically relevant nutrients (Clemens et al. 2002). Therefore, Cd is taken up in a non-specific manner and competes with required ions for binding and uptake. Recently, eight major classes of molecular transporters relevant to metals were reviewed (Hall and Williams 2003), and the authors discussed other genes and transporter families outside these eight classes that may also prove to be important. The current state of knowledge about major transporter classes and genes is reviewed below and summarized in Table 2.5.

2.3.1.1. ZIP family of transporters

The ZIP (ZRT, IRT-like proteins) transporter family was suggested to contain around 85 members found in both prokaryotes and eukaryotes (Hall and Williams 2003). The initial family members to be characterized were the Fe transporter AtIRT1 from *Arabidopsis* through functional expression in yeast (Eide et al. 1996), as well as the Zn transporters ZRT1 and ZRT2 from *Saccharomyces cerevisiae* (Zhao and Eide 1996b; Zhao and Eide 1996a). These discoveries paved the way for subsequent identification of the first plant Zn transporters ZIP1-4 from *Arabidopsis* (Grotz et al. 1998), which were cloned into Zn or Fe deficient *S. cerevisiae* and, upon expression, were unable to rescue the Fe deficient phenotype but did successfully restore growth to the Zn deficient phenotype. Since then, additional members have been discovered in *Thlaspi caerulescens* (Pence et al. 2000) and other plants. ZIP proteins are usually associated with Fe and Zn transport, although manganese (Mn) was suggested to also be a physiologically relevant substrate (Guerinot 2000).

ZIP transporters were also discovered in other species. In the model legume *Medicago truncatula*, a total of seven members of the ZIP family were characterized (Burleigh et al. 2003; Lopez-Millan et al. 2004), although roles in Cd transport were

not investigated. In transformed yeast, MtZIP1 was capable of Zn transport while MtZIPs 5 and 6 were capable of both Zn and Fe transport. MtZIPs 3 and 4 appeared capable of Fe transport and MtZIPs 4 and 7 restored growth of the *smf* yeast mutant that normally exhibited a Mn transport deficiency. RT-PCR results from plant metal experiments generally confirmed that MtZIP transcript levels were responsive to the lack or abundance of various metals, although MtZIP6 expression appeared to be constant under all of the tested conditions (Lopez-Millan et al. 2004). Interestingly, this gene was phylogenetically clustered with Fe transporters, but did not appear to transport Fe in yeast. As usual, care was needed in extrapolating yeast results to metal transporting roles *in planta*, and more work was needed in order to better define roles of these transporters.

Additional ZIP members were found in rice where 10 ZIPs and 1 IRT member were reported (Ishimaru et al. 2005), although once again roles in Cd transport were not studied. Microarray and northern blot results suggested several of the genes were responsive to metal deficiencies through either up- or down-regulation, although the strongest responses were from OsZIP4 under Fe deficient conditions in the roots and shoots, and from OsIRT1 under Fe and Mn-deficient conditions in the roots. Further investigation of OsZIP4 revealed it restored growth of *zrt1zrt2* transformed yeast mutants under Zn-limited growth conditions. OsZIP4::GFP fusions localized to the plasma membrane in onion epidermal cells and in situ hybridization found transcripts present in vascular tissues of Zn-deficient plants, which suggested a possible role in metal translocation (Ishimaru et al. 2005). Furthermore, TjZNT1 and TjZNT2 were cloned from the Ni hyperaccumulator *Thlaspi japonicum* (Mizuno et al. 2005). Yeast transformed with these transporters were found to better tolerate higher Ni levels despite total Ni content that did not exhibit statistically significant differences from controls. Both transporters allowed growth of Mn-deficient yeast, and TjZNT1 was

able to complement a Zn-deficient yeast mutant (Mizuno et al. 2005). Other work on MxIRT1 from *Malus xiaojinensis* found that it was upregulated in Fe-deficient plants and complemented Fe uptake in mutant yeast with localization to the plasma membrane (Li et al. 2006).

Members of the ZIP family exhibited significant divergence when sequence and predicted structure were considered. In general, most members of this family were noted to contain eight transmembrane domains and between 309-476 amino acid residues (Guerinot 2000), although some members were suggested to have only five such domains (Gaither and Eide 2001), which indicated that this feature was somewhat variable. Eng et al. (1998) noted that most of the observed variation between members was in the N-terminal region; these authors also mentioned the possibility of sequencing artifacts producing some of the observed differences. A designated "variable region" exists between transmembrane domains III and IV, which has been found to frequently contain a potential cytoplasmic region that is rich in histidine residues (Guerinot 2000) and may be involved with metal binding or protein-protein interactions. Again, variability was apparent with this feature; Eng et al. (1998) noted that this his-rich region may occur in other parts of the gene, or not at all, as was the case for ZIP2 and ZIP3 from *Arabidopsis*. Another characteristic feature of ZIP members was a highly conserved region in transmembrane domain IV which contains a fully conserved histidine and glycine; this region was suggested to be part of an intramembranous heavy metal binding site (Eng et al. 1998).

Phylogenetic relationships between ZIP family members were initially divided into two main groups based on sequence similarity. Subfamily I included known genes from several plants as well as a protozoan and Subfamily II included genes from higher animals (Guerinot 2000). Gaither and Eide (2001) used more sensitive database searching techniques and added two additional groups: the LIV-1 and gufA

subfamilies, each of which had representatives from a wide range of species. Although all of these subfamilies comprised the total ZIP family, not all were yet particularly well studied. For instance, no members of the LIV-1 subfamily have been functionally characterized (Gaither and Eide 2001). Therefore, database classifications based on sequence similarities have not yet been fully supported by functional and substrate studies of gene members in all cases.

In addition to an incomplete functional understanding, regulation of ZIP members at both the gene and protein levels was not yet well elucidated. Exact control mechanisms were not understood and could possibly differ between some family members based on their physiological roles. Yeast members ZRT1 and ZRT2 were studied by Zhao and coworkers (1998) who found them to be regulated transcriptionally by binding of ZAP1, a Zn-responsive transcriptional activator. ZAP1 was suggested to exert its effects through Zn response elements (ZREs) that were located in the promoter regions of ZRT1, ZRT2, and ZAP1, which indicated the activator also regulated itself (Zhao et al. 1998). Apart from genetic control, once active protein is in place and functioning, fast down regulation may be needed when adequate or excessive ion levels are reached in order to prevent cell damage. Gitan et al. (1998, 2000) established that for ZRT1 in yeast, the transporter is tagged with ubiquitin prior to endocytosis and vacuolar degradation. These authors (Gitan et al. 1998; Gitan and Eide 2000) also demonstrated that a lysine residue, Lys 195, was crucial for ubiquitin attachment and subsequent degradation. Again, differences may exist between family members, and whether this is the same type of mechanism plants use is unclear. However, *Arabidopsis* IRT1 was noted to contain two Lys residues that may possibly serve as sites for ubiquitin attachment (Connolly et al. 2002), which suggested that plant ZIP members may indeed be down-regulated in an analogous

manner. Exact mechanisms of transcriptional control in plants were, unfortunately, poorly understood.

When considering Cd and ZIP transporters, some members have been shown be sufficiently non-selective and able to transport this metal. For instance, several lines of evidence have suggested a role for IRT1 in Cd uptake. Rogers et al. (2000) conducted a detailed study on IRT1 and found it capable of transporting Cd when expressed in yeast. Intriguingly, IRT1 substrate specificity was altered by site-directed mutagenesis, which demonstrated that only a few critical residues can determine a transporter's specificity and thus may have use in future engineering applications (Rogers et al. 2000). Further evidence implicating IRT1 as a route of Cd uptake was provided by an *Arabidopsis* mutant lacking the IRT1 gene (Vert et al. 2002). When plants were grown under Fe-limiting conditions, wild type plants were severely chlorotic and contained five-fold more Cd in the roots than did the IRT1 mutants. Connolly et al. (2002) created a different mutant *Arabidopsis* line that constitutively overexpressed IRT1 and found the mutant was more sensitive to Cd under Fe-limiting conditions when compared to wild type plants. These authors also reported that Cd and Zn were able to down-regulate IRT1 protein levels under Fe-deficient conditions. Other ZIP members besides IRT1 may also have a role in plant Cd uptake and transport. When reporting the isolation of the *Arabidopsis* ZIP1-4 members, Grotz et al. (1998) noted that ZIP1-3 could transport Cd in yeast, and found ZIP2 to be particularly capable of Cd transport. From the Ni hyperaccumulator *Thlaspi japonicum*, TjZNT1 and TjZNT2 were cloned into various mutant yeast strains and both proteins were found to decrease yeast tolerance to Cd (Mizuno et al. 2005). Based on these studies, then, certain ZIP members appeared to have a role in Cd uptake in the plasma membrane and are likely an important pathway for Cd trafficking.

2.3.1.2. P-type ATPases

P-type ATPases are a very large and important family of transport proteins and have been found in all three kingdoms. They are ATP driven pumps that function to transport charged substrates across membranes and are divided into five major classes and several subfamilies based on sequence similarity and transported substrate. In plants, P-type ATPases were noted to have a variety of important functions, including generation of proton gradients (H^+-ATPases), modulation of cytosolic and organelle Ca levels (Ca^{2+}-ATPases), and transport of micronutrients (Zn and Cu ATPases) (Axelsen and Palmgren 2001). This review focuses on the micronutrient transporters, known as P_{1B}-ATPases (also called the CPx-ATPases and heavy metal ATPases) that also have involvement with heavy metal transport (Williams et al. 2000). The plant heavy metal ATPases (HMAs) were recently reviewed by Hall and Williams (2003) and said to be mainly involved with metal ion homeostasis and efflux from the cytoplasm.

In general, all known P-type ATPases have shared several common structural and mechanistic features. These features included the following: formation of a phosphorylated intermediate, inhibition by vanadate, 8-12 transmembrane domains, N- and C-termini on the cytoplasmic side, as well as ATP binding and phosphorylation sites (Axelsen and Palmgren 2001). A review noted that all members of this class of transport protein can be structurally divided into four distinct domains: the P-domain, N-domain, A-domain, and M-domain, all of which perform a mechanistic function (Kuhlbrandt 2004). Additionally, Kuhlbrandt's (2004) review graphically detailed the different conformational states and general catalytic cycle (Figure 6), which provided an excellent general model for how these transporters operate. On top of these universal features, several unique characteristics of P_{1B}-ATPases have been noted: a

heavy metal binding site near the N-terminus, a conserved cysteine-proline-(cysteine, histidine, or serine) or CPx motif, and eight transmembrane domains rather than the usual 10 or 12 (Solioz and Vulpe 1996). A survey of the *Arabidopsis* genome (Axelsen and Palmgren 2001) found 45 total genes coding for P-type ATPases and seven genes specifically in the P_{1B} subclass; notably, this number was the highest ever found in any one organism and indicated the importance of these proteins to plants. Further investigations later found an additional P_{1B} member in *Arabidopsis*, which brought the total to eight (Cobbett et al. 2003; Colangelo and Guerinot 2006; Argüello et al. 2007).

Like other organisms, genetic analysis of *Arabidopsis* P_{1B} members showed clustering into two groups based on what ion was likely transported: the Zn/Co/Cd/Pb subclass and the Cu/Ag subclass (Cobbett et al. 2003). More detailed sequence analysis led to the division of P_{1B}-ATPases into six subgroups of different apparent specificities based on conserved sequences within each subgroup (Argüello 2003). Transport specificities were divided into Cu/Ag (Group 1B-1), Zn/Cd/Pb (Group 1B-2), Cu/Cu/Ag (Group 1B-3), Co (Group 1B-4) with groups 1B-5 and 1B-6 of undetermined specificity. However, exact physiological roles and substrate specificities for many members of these subgroups were not yet experimentally established, and thus full verification of this *in silico* analysis was not possible.

Several P_{1B} members have now been studied, generally through transformation into yeast or through isolation of gene knockout lines in *Arabidopsis*. In plants, Hirayama et al. (1999) first isolated an *Arabidopsis* mutant RAN1 (AtHMA7) that displayed an ethylene response phenotype when exposed to trans-cyclooctene (TCO), which normally acts as an ethylene response inhibitor. The gene responsible for this observed phenotype was isolated and termed RAN1; upon further characterization this gene was found to be a Cu-transporting P-type ATPase and was able to complement a

yeast CCC2 mutant that was deficient in Cu transport (Hirayama et al. 1999). RAN1 was thus postulated to transport Cu in a post-Golgi compartment whereby the ion was used to create functional ethylene receptors (Hirayama et al. 1999).

HMA4 from *Arabidopsis* was the subject of several studies. The first group to report on this gene suggested a possible role in Zn transport since it was able to complement an *E. coli* mutant, *zntA*, that was deficient in Zn efflux capability (Mills et al. 2003). Notably, yeast expressing this gene also exhibited enhanced Cd tolerance to the range of metals that was tested (0 – 120 nM). By using RT-PCR, AtHMA4 expression was found in all analyzed plant tissues, although highest levels were found in roots and the gene appeared to be upregulated by Zn and Mn but down-regulated by Cd (Mills et al. 2003). The authors suggested AtHMA4 may have an efflux function in plants or a possible role in xylem loading for metal delivery to the shoot. More recent work on AtHMA4 by the same group found that *Arabidopsis* mutants lacking HMA4 appeared to be more sensitive to Zn and Cd based on reduced fresh and dry weights when grown in elevated concentrations of either of these metals (Mills et al. 2005b). Additional yeast expression experiments found that Zn sensitive *zrc1 cot1* yeast phenotypes were also rescued, further supporting a role in Zn transport. Interestingly, a truncated version of the gene lacking the last 457 amino acids led to even higher tolerance of both Cd and Zn in yeast, which suggested some kind of regulatory function for this domain. To explain their results in both yeast and plants, Mills et al. (2005) proposed a dual function model for AtHMA4 that included xylem loading and detoxification roles *in planta*.

Additionally, AtHMA4-GUS fusions were found localized to several tissues, including the vascular tissue in roots, leaves, and stems (Hussain et al. 2004; Verret et al. 2004). Metal content analysis of wild type, null, and overexpressing *Arabidopsis* mutants was unable to conclusively establish a role in vascular transport, however

(Verret et al. 2004). In leaves, null mutants appeared to have lower levels of Cd and Zn, although not all of the results were statistically significant. Roots showed generally higher accumulations of Zn and Cd in the null mutants, but again results were not always statistically significant. In overexpressing mutants, leaves generally had higher levels of Zn and Cd, while roots showed no difference in metal content between wild type and overexpressing mutants. Also, better plant growth at high metal concentrations (Zn, Cd, and Co) was observed at high levels of AtHMA4 expression (Verret et al. 2004). Additional work in yeast confirmed that AtHMA4 was capable of transporting Zn, Cd, Pb, and Co and expression led to increased yeast tolerance to all of these metals except Co (Verret et al. 2005).

Other work on AtHMA4 in yeast, however, produced conflicting results. Mutations of two cysteine residues in the N-terminus resulted in nonfunctioning transporters, which indicated these amino acids were important for proper function of AtHMA4. Likewise, deletion of a stretch of 11 histidines in the C-terminal section also resulted in a nonfunctioning transporter (Verret et al. 2005), which contrasted with the Mills et al. (2005) truncated gene experiment. In working with AtHMA4 and the *Thlaspi caerulescens* homolog TcHMA4, Bernard et al. (2004) reported increased sensitivity in two yeast strains that expressed the full length protein, but higher tolerance in one yeast where a truncated version was expressed. These results may have been due to innate metal uptake and protein localization differences between the strains (Bernard et al. 2004), as their report conflicted with others who found increased tolerance to Cd, Zn, and Cu in yeast expressing TcHMA4 that was likely caused by metal efflux from the cell (Papoyan and Kochian 2004). Taken together, a role in metal trafficking, efflux into the apoplast, and xylem loading all appeared as possible roles for AtHMA4 in *Arabidopsis*, but further work was needed in establishing the function(s) of protein regulatory domains.

Roles for other P_{1B} members were investigated as well, and at least three members appeared to be important for Cu transport to the chloroplast. Among these were PAA1, also called HMA6 (Shikanai et al. 2003). Mutant plants were found to display a high chlorophyll-fluorescence phenotype that could be rescued by addition of 5 or 10 μM $CuSO_4$ to the growth media. Furthermore, based on several lines of evidence that included confirmation of a chloroplast targeting sequence, reduced electron transport rates, significantly lower nonphotochemical quenching induction, and low Cu content of chloroplasts, a role of transporting Cu across the plastid envelope was proposed (Shikanai et al. 2003). PAA2 (HMA8) was later isolated in *Arabidopsis* by the same laboratory and radiolabeling experiments as well as GFP fusions to the first 306 residues of this protein strongly indicated a localization to the thylakoid membranes in chloroplasts (Abdel-Ghany et al. 2005). Like PAA1, null mutants exhibited a high fluorescence phenotype that was rescued by addition of $CuSO_4$ to the growth media. Furthermore, metal content analysis revealed that thylakoids in mutant plants contained significantly less Cu than their wild-type counterparts, which led to the suggestion that PAA2 functioned in Cu delivery to the thylakoids (Abdel-Ghany et al. 2005). In addition to PAA1 and PAA2, a study on AtHMA1 suggested it also had a role in Cu delivery to the chloroplast (Seigneurin-Berny et al. 2006). Null mutants had sharply reduced chloroplast Cu levels and displayed a highly photosensitive phenotype when grown under high-light conditions which could not be rescued by addition of Cu to the growth media. GFP fusions and western blots indicated a chloroplast envelope localization, although RT-PCR analysis found detectable transcript in roots, which possibly indicated additional roles for this transporter. In yeast, expression of AtHMA1 resulted in Cu and Zn hypersensitivity, although *in planta* evidence pointed to Cu as the primary substrate. Clearly, AtHMA1

and PAA1 were not functionally redundant (Seigneurin-Berny et al. 2006), and further clarification of their exact roles was needed.

Although not apparently associated with chloroplasts, *Arabidopsis* HMA5 was another P_{1B} member that appeared to be involved mostly in Cu trafficking. Work by Andres-Colas and colleagues (2006) found HMA5 transcripts mainly in roots and flowers, with some expression in above-ground plant tissue. Null mutants were hypersensitive to Cu, and to a lesser extent, Ag, and root metal content in these plants was found to be higher than in wild-type plants, despite shoot metal levels that were unchanged. Interestingly, yeast assays suggested an interaction with plant Cu chaperones ATX1 and CCHD, and Cu detoxification and compartmentalization was suggested as the primary role for HMA5 (Andres-Colas et al. 2006). Verification for these interactions *in planta* as well as localization experiments were needed, however, before a biological role could be more defined.

Other P_{1B} members were less understood. A report on *Arabidopsis* HMA3 found that Cd, and possibly Pb, tolerance in the Cd-sensitive *Δycf1* yeast strain was enhanced by HMA3 expression (Gravot et al. 2004). Zn tolerance, though, in the Zn-sensitive *Δzrc1* strain was not enhanced, and HMA3 expression in a wild type strain failed to increase tolerance to any of the three metals. GFP fusion protein experiments failed to reveal where HMA3 localized in yeast, and while mRNA analysis of *Arabidopsis* found detectable levels of transcript throughout the plant, they were apparently unresponsive to short or long term heavy metal exposure. In a study of Arabidopsis HMA2, Eren and Argüello (2004) found that a variety of metals elicited ATPase activity in vesicles isolated from yeast that were transformed with HMA2. Direct evidence for Zn transport in vesicles was presented, although no other metals were apparently tested. Homozygous *Arabidopsis* HMA2 knockout mutants showed no obvious phenotype, although whole plant Zn levels were higher in these plants

when grown under normal or high Zn conditions. Cd levels were also found to be higher when grown at very elevated (125 μM) Cd concentrations. RT-PCR analysis found HMA2 transcript in roots, leaves, stems, and flowers that was apparently unresponsive to high metal concentrations (Eren and Argüello 2004). Other work found that HMA2-GUS fusions followed a nearly identical expression profile to HMA4: vascular tissue in roots, leaves, and stems showed staining, and HMA2::GFP fusion proteins localized to the plasma membrane in *Arabidopsis* (Hussain et al. 2004). Since HMA4 HMA2 double knockout mutants (but not single knockout lines) showed a severely chlorotic and stunted phenotype, these P_{1B} members appeared to share at least some degree of functional redundancy and further experiments were needed to clarify their biological roles.

2.3.1.3. Nramp transporters

Nramp (natural resistance associated macrophage proteins) transporters represent another major class of membrane transport protein in plants. These proteins have been found in a diverse range of organisms from plants and animals to bacteria and fungi (Hall and Williams 2003). The first member of this family, Nramp1, was found in mammals and research revealed it to be involved with phagocytosis by regulating divalent ion concentrations in the phagosome that in turn harms bacterial replication (Williams et al. 2000). Recent reviews underscored the importance of NRAMP proteins to animal life by noting that defects in Nramp2 were linked to severe anemia and even death in rats and humans due to defects in Fe transport (Courville et al. 2006; Nevo and Nelson 2006). Thus, NRAMP transporters have been the subjects of intense study in animals. In plants, six members have been identified in *Arabidopsis* (Maser et al. 2001), eight in rice (Gross et al. 2003), and one in

soybean (Kaiser et al. 2003). Phylogenetically, these genes were found to segregate into two main subfamilies in *Arabidopsis*: AtNRAMPs 2-5 in one group and AtNRAMP1 and AtNRAMP6 in the other group (Maser et al. 2001). The rice genes were also found to display a similar clustering pattern (Curie et al. 2000), which further reinforced these groupings.

Structurally, plant members were not very different from other Nramp proteins: they were predicted to have 12 transmembrane domains and a signature consensus transport motif (CTM) between transmembrane domains 8 and 9 (Curie et al. 2000; Williams et al. 2000). The CTM region exhibited a high degree of conservation between plant and animal members, and was suggested to interact with ATP-coupling subunits (Curie et al. 2000). This was at odds, though, with the apparent need for H^+ ions for metal transport, which indicated NRAMPs relied upon H^+ gradients as an energetic source, rather than ATP (Courville et al. 2006; Nevo and Nelson 2006). Further mechanistic studies on rat DCT1 and yeast Smf1p revealed an uncoupled slip of H^+ ions, or in the case of Smf1p, Na^+ ions that was a fundamental part of their function (Nevo and Nelson 2006). However, the biological reasons behind such uncoupled transport were not understood and whether or not this phenomenon was common to all NRMAPs was unknown. Likewise, exact mechanisms of transport, control, and physiological functions in plants were largely unclear, although preliminary studies revealed some apparent roles for plant NRAMP members.

Curie et al. (2000) cloned AtNRAMPs 1 and 2 into Fe transport deficient yeast and found that AtNRAMP1 but not AtNRAMP2 was able to restore growth at relatively high Fe concentrations. Additionally, these authors studied expression patterns in *Arabidopsis* and found AtNRAMP1 mRNA at low levels throughout the plant which was up-regulated under Fe starvation; alternatively, AtNRAMP2 mRNA

was expressed constitutively at high levels in roots and low levels in shoots, but was slightly down regulated by Fe starvation. Overexpression of AtNRAMP1 yielded plants that were able to grow at higher external Fe concentrations than wild-type controls, which suggested a role for this gene in Fe homeostasis (Curie et al. 2000). Other investigators (Thomine et al. 2000) demonstrated that AtNRAMPs 1, 3, and 4 may have broad substrate specificities and the ability to transport Cd. Thomine et al. (2000) found that AtNRAMPs 1, 3, and 4 were able to complement Mn transport deficient yeast in addition to Fe-deficient mutants, and they found yeast strains expressing these genes had a higher sensitivity to Cd. Plants disrupted in AtNRAMP3 had higher resistance to Cd while AtNRAMP3 overexpressing lines were more sensitive to Cd and accumulated more Fe when grown on minimal media in the presence of Cd; all three genes were up-regulated under low metal conditions, although in some cases expression was relatively tissue specific (Thomine et al. 2000).

More recently, AtNRAMP3 was found to localize to vascular tissues in roots, stems and leaves under Fe-sufficient conditions and was suggested to be involved in long distance metal transport (Thomine et al. 2003). GFP fluorescence studies revealed the protein was localized to the vacuole, and based on gene knockout as well as overexpressing mutants, Thomine et al. (2003) proposed AtNRAMP3 served to remobilize Fe from the vacuole under Fe-limiting conditions. Further work on AtNRAMP4 established that it had the same expression profile as AtNRAMP3 and was active during early seed germination (Lanquar et al. 2005). AtNRAMP4 was upregulated in response to Fe deficiency, and NRAMP3 NRAMP4 double knockout mutants were stunted, bleached, and had a high mortality rate (> 90%) when grown on calcareous soil. Mutant seeds contained significantly higher amounts of Fe in their seed vacuole globoids than wild-type plants, and thus AtNRAMP3 and AtNRAMP4

were suggested to have redundant roles in mobilizing Fe from the vacuole globoids during early developmental stages (Lanquar et al. 2005).

In soybean, Kaiser and colleagues (2003) isolated GmDMT1, a soybean Nramp homolog and found that it was localized to the peribacteroid membrane of soybean nodules, although Northern blots detected weak RNA signals in leaves, stems, and roots as well. Functional complementation in yeast suggested this transporter had the ability to transport Fe and Zn, while excess concentrations of Mn, Zn, and Cu were found to inhibit Fe uptake. Thus, GmDMT1 was suggested to serve as a Fe(II) transporter in soybean symbiosomes (Kaiser et al. 2003). Other work in rice was done that indicated OsNRAMPs 1-3 may be involved in various aspects of plant defense response and pathogen interactions (Zhou and Yang 2004). OsNRAMPs 1-3 showed little or no response to a virulent strain of *Magnaporthe grisea*, a fungal pathogen that causes rice blast disease, but all were upregulated in response to infection by an avirulent strain of this pathogen that carried the *avrPita* gene. Infection by *Burkholderia glumae*, a bacterial pathogen, resulted in upregulation of OsNRAMPs 2 and 3. Furthermore, up- or down-regulation of the rice NRAMPs was observed under some treatments of salicylic acid, jasmonic acid, and abscisic acid (Zhou and Yang 2004), although results differed between plant and cell suspension cultures, which made interpretation difficult. Thus, more incisive studies appeared necessary before conclusive roles in plant defense could be established, and work was clearly needed before a better mechanistic understanding of plant NRAMP mode of action could be achieved, as well as the relative importance of this class to Cd transport.

2.3.1.4. Cation diffusion facilitator (CDF) or cation efflux (CE) transporters

The CDF family of transporters was another transporter family involved with heavy metals. Several members of this family have been found in animals and microorganisms (for reviews, see Maser et al. 2001, Hall and Williams 2003, Haney et al. 2005), from which initial information on structure and specificity were derived. The first plant member isolated and partially characterized was ZAT from *Arabidopsis* (van der Zaal et al. 1999), which Maser et al. (2001) proposed be re-named AtMTP1, for Metal Tolerance Protein 1. Initial analysis of the *Arabidopsis* genome revealed eight members of the CE family (Gaither and Eide 2001; Maser et al. 2001), although this number was later revised upwards to include 12 total members (Blaudez et al. 2003). Phylogenetically, the CE family was found to cluster into four distinct groups (Maser et al. 2001; Blaudez et al. 2003; Montanini et al. 2007), that were divided into Fe/Zn transporters, Zn-only transporters, and Mn CDF transporters (Montanini et al. 2007). Based on genetic data, CE members were found to have six transmembrane domains, a unique signature sequence, and significant size variability, particularly between the latter transmembrane domains as well as the C-termini (Paulsen and Saier 1997; Haney et al. 2005). Additionally, eukaryotic members were found to have a histidine-rich region between transmembrane domains four and five that was suggested to be a metal binding domain that served a functional or regulatory role (Paulsen and Saier 1997).

The first plant identified plant member, ZAT or AtMTP1 from *Arabidopsis*, was isolated by van der Zaal (1999). They found the gene was constitutively expressed and that overexpression yielded plants with modestly higher Zn tolerance with higher Zn levels in roots. However, Cd tolerance was unaltered, but due to the modestly enhanced Zn tolerance, the authors proposed ZAT had a role in vacuolar

sequestration. Additional work (Bloss et al. 2002) on this gene discovered that when expressed in proteoliposomes, it was able to transport Cd at 1% the rate of Zn and did not require a proton driving force, although the authors noted that data from expression in *Ralstonia metallidurans* fitted well with 100 mV-driven uniport. They also found ZAT to function as an uptake system, which led them to suggest that CE members could transport cations in both directions, dependent upon protein, cation, and substrate concentration (Bloss et al. 2002). Later, based on evidence from western blots, sucrose density gradients, and AtMTP1::GFP fusion proteins, a vacuolar localization was confirmed for this transporter (Kobae et al. 2004). Furthermore, these authors created a T-DNA insertion mutant and found that while no phenotype was evident when grown under normal conditions, exposure to high levels of Zn revealed a hypersensitive phenotype with reduced root length and changes in cell morphology. When expressed in *Xenopus laevis* oocytes, AtMTP1 appeared to result in higher oocyte Zn levels than controls when exposed to 0.1 mM Zn, and lower Zn levels than controls when placed in media with trace Zn amounts (Desbrosses-Fonrouge et al. 2005). However, endogenous transport systems may have been responsible for the conflicting results. These authors also reported a vacuolar localization for AtMTP1::GFP fusion proteins as well as an insensitivity of transcript levels to Zn and other heavy metals, including Cd (Desbrosses-Fonrouge et al. 2005). Taken together, MTP1 in *Arabidopsis* appeared to have a role in basal Zn tolerance mechanisms by transporting cytosolic Zn into the plant vacuole, although questions about possible cell-specific roles remained (Kramer 2005).

Other CDF members in *Arabidopsis* were also studied. AtMTP3 was cloned and expressed in the Zn-sensitive *zrc1 cot1* yeast mutant and eliminated the Zn sensitivity of this strain; however Cd, Fe, and Mn sensitivities were unaffected and Co tolerance was only slightly increased, which suggested an overall strong Zn transport

preference (Arrivault et al. 2006). Like MTP1, AtMTP3::GFP fusion proteins were localized to the plant vacuole and RT-PCR showed that transcript levels were an order of magnitude higher in seedling roots than in other parts of the plant. Interestingly, root transcript levels were noted to be responsive to Zn and Co, while responses to Mn were observed in times of Fe-deficiency. Additionally, RNAi plant lines were generated and found to be sensitive to high Zn levels and showed reduced shoot weights under these conditions. Given that MTP1 and MTP2 transcript levels were not significantly reduced in these RNAi lines (Arrivault et al. 2006), AtMTP3 appeared to have a distinct role from AtMTP1.

Recently, two reports were published on AtMTP11 that found it to be involved in Mn transport (Delhaize et al. 2007; Peiter et al. 2007). Delhaize and colleagues (2007) found that AtMTP11 rescued the Mn sensitivity of the pmr1 yeast strain. Furthermore, dye experiments in yeast vesicles expressing either AtMTP11 or MTP8 from *Stylosanthes hamata* showed that Mn, but not Cd or Zn, rapidly dissipated the proton gradient which suggested these two transporters functioned as H^+/Mn^{2+} antiporters. Both T-DNA insertion mutants and RNAi plant lines were more sensitive to elevated Mn concentrations, and AtMTP11::GFP fusion proteins expressed in tobacco and leek protoplasts showed localization to pre-vacuolar compartments (Delhaize et al. 2007). Another report on *Arabidopsis* MTP11 by Peiter and colleagues (2007) agreed with these results; they found that both yeast cells and mesophyll protoplasts that were transformed with an AtMTP11::GFP fusion protein displayed a punctate fluorescence pattern that, in protoplasts, overlapped the fluorescence pattern of a trans-Golgi marker. RT-PCR results showed no evidence for up- or down-regulation of MTP11 transcript in response to long or short-term Mn exposure. Plants that overexpressed AtMTP11 had a harder time growing on regular plant media, but could grow well on media containing 1.5 mM Mn, which was toxic to

wild-type plants (Peiter et al. 2007). Taken together, these papers provided evidence for a Mn tolerance mechanism that relied upon AtMTP11 to transport excess cytosolic Mn into secretory vesicles. This type of a mechanism was previously undescribed in plants, and it was unknown if a similar strategy was used for other metals beside Mn.

Another CE gene was isolated by Persans et al. (2001) from the hyperaccumulator *Thlaspi goesingense* that yielded two distinct splice variants that differed in their abilities to confer metal tolerance to metal-sensitive yeast strains. TgMTP1t1 conferred the highest tolerance to Cd, Co, and Zn, while TgMTP1t2 conferred superior Ni resistance. Notably, TgMTP1t2 was found to lack most of the central histidine rich domain, which appeared to confer Ni-specificity to this gene. Northern analysis revealed these genes to be constitutively expressed at high levels in shoots of *T. goesingense*, and they were suggested to explain part of the ability of this species to accumulate high shoot Ni levels through high vacuolar sequestration (Persans et al. 2001). A CE member from hybrid poplar, PtdMTP1, was also isolated and characterized through expression in yeast and creation of PtdMTP1-GFP fusion proteins (Blaudez et al. 2003). The gene was constitutively expressed in most plant tissues with highest expression levels found in mature roots and leaves. Additionally, PtdMTP1 rescued Zn sensitivity in yeast, but not other heavy metal sensitive mutants and GFP fusion proteins showed it localized to the vacuole in yeast and plants. Thus, clear evidence for a role in vacuolar transport was found for this gene, although experiments focused on understanding the energetics of this transporter did not reveal a definitive mechanism. Using yeast defective in vacuolar ATPase driven acidification, Blaudez et al. (2003) found that PtdMTP1 partially rescued the metal sensitive phenotype, which indicated that this transporter did not require a H^+ gradient for operation. The authors, however, cautioned that the same results could have been obtained if PtdMTP1 was present on non-vacuolar endomembranes that did not rely

on vacuolar ATPase activity for acidification, and therefore the energetics of this protein were not clear. MTP1 was also isolated from *Nicotiana glauca* and *Nicotiana tabacum*; genes from both species were found to alleviate the Zn sensitivity of the zrc1 yeast strain (Shingu et al. 2005). GFP fusion proteins were localized to the yeast vacuole, and analysis of plant RNA transcripts suggested similar expression levels across both species. Also, in Zn-sensitive vph2 and vma8 yeast that were unable to acidify the vacuole, both NgMTP1 and NtMTP1 genes were able to allow growth on Zn-containing medium, or, in the case of vph2, Zn- and Co-containing medium (Shingu et al. 2005). Since these yeast were apparently unable to provide the proton gradient needed for a H^+/Zn^{2+} antiport mechanism, this study may have provided some indirect evidence for the uniport mechanism of AtMTP1 suggested by Bloss et al. (2002). However, as noted by Blaudez et al. (2003), a localization to non-vacuolar endomembranes could not be eliminated.

2.3.1.5. Cation/H^+ antiporters

This class of transporters is a large and possibly important one when considering Cd transport in plants, but detailed understanding of this class as it pertained to Cd transport was limited. However, they all share a common feature in that members are expected to use the membrane ATPase generated proton gradient as a driving force for substrate transport. Much of the knowledge about this class of transporter was derived from certain well-characterized Ca^{2+}/H^+ and Na^+/H^+ antiporters in yeast (for review, see Maser et al. 2001), but plant members of this family are not yet well understood. In *Arabidopsis*, more than 40 genes belonging to this family were found, with their encoded proteins predicted to have between 10 and 14 transmembrane domains (Maser et al. 2001). Phylogenetic analysis of this family

by Maser and colleagues (2001) divided members into five groups and *Arabidopsis* was found to contain at least one member from each family, although one family, CPA2, had vastly more members than the others, which may be indicative of its importance to plants.

Although most plant members were not well understood in terms of their possible involvement in Cd transport, a few studies have found some members capable of transporting this metal. The isolation of *Arabidopsis* CAX2 (for calcium exchanger 2) and subsequent expression in yeast resulted in enhanced Mn tolerance, but resistance to other metals, including Cd, was unchanged (Hirschi et al. 2000). CAX2 protein was found at low levels throughout the plant, was unresponsive to elevated metal levels, and cofractionated with the vacuolar membrane on a sucrose gradient. Furthermore, overexpression of CAX2 in tobacco resulted in 3-fold higher root Cd levels (with a slight increase in stem Cd levels) and isolated tobacco tonoplasts displayed higher rates of Cd, Ca, and Mn transport. Hirschi et al. (2000) noted CAX2 had an apparently higher affinity for Cd and Ca over Mn. Other investigators also isolated an *Arabidopsis* homolog (AtCAX2) and, like Hirschi et al. (2000), found that it resulted in enhanced yeast Mn tolerance, but it did not extend to other heavy metals (Schaaf et al. 2002). These authors noted that the two cDNAs they isolated and cloned were truncated in the N-terminal region and expression of the full-length CAX2 gene did not alleviate Mn sensitivity in mutant yeast, which indicated the N-terminal region performed some kind of an autoinhibitory function.

The importance of the N-terminal region in protein activity was further demonstrated in other CAX members by extensive protein engineering and site directed mutagenesis of AtCAX1, AtCAX3, and the mung bean VCAX (Pittman et al. 2002). By creating unique chimeric proteins, the authors found that all of the CAX members had a regulatory N-terminal domain, but regulatory regions were not

interchangeable. They proposed that such domains interacted specifically with their respective transporters, and the slight differences among domains were able to effect inhibition (Pittman et al. 2002). Another member of the cation/H^+ antiporter family was found through homology to a mammalian Na^+/Ca^{2+} exchanger and subsequently dubbed AtMHX1 (Shaul et al. 1999). AtMHX1 was found localized to the vacuolar membrane of plants and was expressed in vascular cylinder tissues of roots, stems, and leaves. This gene appeared to code for a Mg or Zn^{2+}/H^+ exchanger that was suggested to function in loading and unloading capacities of the xylem in xylem parenchyma cells (Shaul et al. 1999). Whether this gene had the ability to transport Cd in plants was unknown since the investigators did not apparently test for transport of this metal, but it may be a promising candidate for xylem loading of Cd as well. Since many members of the cation/H^+ antiporter family were found in the *Arabidopsis* genome (Maser et al. 2001) and relatively few were well-characterized, more information about the roles these proteins may play in plant Cd transport will require further study.

2.3.1.6. CNGC transporters

Cyclic nucleotide gated channels (CNGC) are a class of transporter that may also have some involvement with heavy metal transport in plants. Unlike some other classes of metal transporters, CNGC members have been found in plants and animals, but not unicellular organisms (Talke et al. 2003). An analysis of the *Arabidopsis* genome discovered that 20 members exist in this plant; these were divided into five distinct groups, with Group IV further subdivided into two subfamilies, and thus the CNGCs constitute a major class in *Arabidopsis* (Maser et al. 2001). In their review, Talke et al. (2003) noted that plant CNGCs have a predicted structure containing six transmembrane domains, a novel P-loop selectivity filter, and both a cyclic nucleotide

binding domain (CNB) and a calmodulin binding domain (CaMB) located near the C-terminus, which differed from N-terminal CaMB placement observed in animal members. Thus, calmodulin (CaM) could have different regulatory mechanisms in plant and animal CNGCs. In general, plant CNGC models have suggested activation by cyclic nucleotides (such as cAMP or cGMP), which then allows passage of mono- or divalent ions, and deactivation through binding of Ca-containing CaM (Arazi et al. 2000; Talke et al. 2003).

Although many plant CNGC members exist, relatively few were cloned and thoroughly characterized. Additionally, the role they may play in Cd transport was found to be unclear as no distinct role of CNGC involvement in Cd transport *in planta* had yet been demonstrated. Despite this, members of this class have shown the ability to transport heavy metals. Arazi and colleagues (1999) isolated a CNGC member from tobacco and dubbed it NtCBP4. They found the expressed protein co-fractionated with the plasma membrane in a sucrose gradient and that protein blots were capable of binding CaM. Furthermore, overexpression of NtCBP4 in tobacco revealed transgenic phenotypes that were more tolerant to Ni but less tolerant to Pb in hydroponic culture. Importantly, responses to other heavy metals, including Cd, were tested but response in overexpressing plants was not found to differ from that of wild type plants (Arazi et al. 1999). Additional work (Sunkar et al. 2000) demonstrated that overexpression of a truncated version of this protein that lacked the entire CaMB domain and part of the CNB domain resulted in tobacco plants that were more tolerant to high levels (> 0.1 mM) of Pb than controls. This indicated the importance of the C-terminal regulatory domains for activity. Again, no evidence for an altered Cd response was found. The authors also created transgenic *Arabidopsis* knockout lines lacking the *Arabidopsis* homolog of NtCPB4, called AtCNGC1, and the resulting plants were again somewhat more tolerant to high Pb levels.

However, heavy metal transport may not be the primary role of this transporter. Another report on AtCNGC1 showed that mutant plants lacking this gene exhibited a small, but statistically significant, decrease in Ca accumulation (Ma et al. 2006). Yeast experiments confirmed the ability to transport Ca, and further characterization of the AtCNGC1 plant mutants found an altered root gravibending response to gravistimulation. Mutant plants also exhibited a longer root phenotype than their wild type counterparts; interestingly, this trait was remedied through lowering media Ca concentrations (Ma et al. 2006). The authors suggested this protein may have a role in Ca uptake or signaling pathways, but the exact mechanism(s) and biological role(s) remained unclear. Additional work in yeast by this laboratory (Ali et al. 2006) found that AtCNGC1M2, a construct with a portion of the CaM binding domain eliminated, was capable of complementing potassium (K) uptake-deficient yeast. However, Ma et al. (2006) found no statistically significant difference in K accumulation between AtCNGC1 knockout and wild type plants, thus failing to confirm an *in planta* K transport role for AtCNGC1.

Other plant CNGCs appeared to have a role in plant defense responses. Characterization of an *Arabidopsis* mutant that was defective in AtCNGC4 expression revealed mutant phenotypes that consisted of numerous leaf lesions surrounded by autofluorescent phenolic compounds that were normally characteristic of plant defense responses (Balague et al. 2003). Additionally, mRNA transcript levels of several known plant defense response genes were abnormally high, and plants further displayed no hypersensitive response to inoculation with various pathogens. Although no experiments on transport specificities were performed, GUS fusions showed a transient increase of AtCNGC4 expression in response to infection (Balague et al. 2003). Collectively, these results suggested that at least one role of AtCNGC4 was in plant response to pathogen infection. Work on AtCNGC11 and AtCNGC12 single-

knockout lines showed they more were susceptible to certain pathogen infections (Yoshioka et al. 2006). Likewise, mutants lacking AtCNGC2 exhibited a severely Ca sensitive phenotype that resulted in drastically stunted growth when moderate concentrations (10-30 mM) of Ca were used in the growth media (Chan et al. 2003). These plants also failed to develop a hypersensitive response to pathogen infection, which suggested some importance of this transporter in that response pathway. Later work found that AtCNGC2 transported Ca into plant cells and was important in plant perception of pathogen attack by providing a Ca current that resulted in downstream nitric oxide production which resulted in initiation of plant immune responses (Ali et al. 2007).

The exact biological roles of other CNGCs were less clear, although they did not appear to have a role in plant defense. Work on CNGC3 in *Arabidopsis* found that T-DNA knockout lines had lower germination rates in the presence of elevated Na levels, but in later stages of development were capable of better growth than WT plants when exposed to elevated levels of K or Na (Gobert et al. 2006). Notably, mutant resistance to Cd and other heavy metals remained unaltered. Cloning in yeast suggested AtCNGC3 was capable of transporting both Na and K while GUS fusion constructs showed early expression in root tissues followed by rising expression levels in shoots. AtCNGC3::GFP fusion proteins localized to the plant plasma membrane, and thus AtCNGC3 appeared to have a role in monovalent cation uptake in roots. Another CNGC from *Arabidopsis*, AtCNGC10, was expressed in an *E. coli* mutant defective in K uptake and was able to allow growth at lower K concentrations than vector controls (Li et al. 2005). When transformed into yeast, the same effect was observed; antisense plant lines that were constructed contained an average of 40% less K than wild type controls grown under the same conditions. Further work by the same laboratory used AtCNGC-specific antisera and immuno-electron microscopy on leaf

sections to localize AtCNGC10 to the plasma membrane of parenchyma and mesophyll cells (Borsics et al. 2007). In antisense plants, leaf surface area and lamina thickness were reduced, and leaf starch content was nearly doubled. Furthermore, root lengths were reduced 38% and plants were more sensitive to K scarcity. Although precise biological function in *Arabidopsis* was unclear, the authors speculated AtCNGC10 may be involved in modulating K^+ fluxes in a light-signaling pathway (Borsics et al. 2007).

Clearly, most CNGCs studied did not appear to have a direct role in heavy metal uptake, trafficking, or tolerance. Given that many members of this class were not yet studied or well-characterized, a possible role in heavy metal transport could not be eliminated. Therefore, while many plant CNGC members were suggested to be involved with ionic (Ca, K, etc.) and cyclic nucleotide signaling pathways (Arazi et al. 2000; Talke et al. 2003), at least some CNGC members had ability to transport divalent heavy metals as well. More studies on this transporter class were needed before the likelihood of their involvement in plant heavy metal pathways could be more accurately assessed.

2.3.1.7. Multidrug and toxin efflux family (MATE)

The MATE family has been found to be another class of transporter that may be involved in Cd transport within plants. However, relatively little information about plant MATE proteins has been reported. The first plant member of this family, AtDTX1, was discovered in 2002 through screening of an *Arabidopsis* cDNA library and functional complementation of an *E. coli* mutant susceptible to norfloxacin (Li et al. 2002). These authors then conducted database searches of the *Arabidopsis* genome and found an additional 56 members of this family that were phylogenetically divided

into five groups. Plant MATE members were predicted to generally have 12 transmembrane domains, although one of the families exhibited some variability in this feature. Overexpression of AtDTX1 in *E. coli* resulted in a strain capable of detoxifying norfloxacin and the ability to tolerate 10-fold higher Cd concentrations; AtDTX1 mRNA was found in all plant tissues, although more predominately in flowers and stems; GFP fusion proteins appeared to be localized to the plasma membrane (Li et al. 2002). Thus, the protein appeared to be a plasma membrane transporter with broad substrate specificity, and was suggested to be a plant toxin efflux protein. However, no direct evidence supporting this role in plants was presented and further work elucidating the role of this gene was lacking.

A couple other members of the MATE transporter family were also characterized. *Arabidopsis* FRD3 was isolated in mutant plants that displayed a Fe-deficient response even in the presence of adequate Fe and were found to contain higher metal levels than wild type plants (Rogers and Guerinot 2002). Mutants were found to express high levels of FRD3, particularly under Fe-sufficient conditions, and the gene was thought to be involved in Fe homeostasis, although an exact role was not established (Rogers and Guerinot 2002). Like AtDTX1, FRD3 was predicted to have 12 transmembrane domains and was expected to localize to the plasma membrane based on genetic sequence. Later work found that xylem sap of plants lacking FRD3 contained less citrate and Fe than WT controls, and voltage clamping experiments on *Xenopus* oocytes showed an inwardly directed current when citrate was in the external bathing medium (Durrett et al. 2007). Additionally, plants that ectopically expressed FRD3 showed enhanced tolerance to aluminum and contained higher amounts of citrate in their roots than WT plants, which led Durrett et al. (2007) to suggest that FRD3 transported citrate into the xylem. Another MATE member, called *Arabidopsis thaliana transparent testa12* (TT12) was recently isolated and characterized as a

tonoplast protein that transported flavonoids into the vacuole (Marinova et al. 2007). Thus, biological roles and substrate specificities of MATE members appeared to be diverse in plants, with some members of this family potentially involved in plant Cd transport.

2.3.1.8. Other transporters

In addition to the transporters mentioned previously, plants have been found to contain additional transporter families that may be involved with heavy metal transport. A Cu transporter family has been found in *Arabidopsis* that consisted of five unique members called COPT1-5 (Sancenon et al. 2003). These genes contained three transmembrane domains and generally appeared to be involved in Cu transport and homeostasis within the plant. RT-PCR analysis showed COPT1-5 was expressed in many plant tissues, although some of the genes showed preferential expression in certain tissues. Using metal competition studies, COPT1 was unable to transport Cd but 25-fold excesses of Ag and Mn reduced Cu transport by 83% and 30% respectively, showing that this transporter was not entirely specific for Cu (Sancenon et al. 2003). The metal specificities of COPT2-5 were not examined, and thus questions remained about the possible involvement of this family in Cd transport.

Another transporter family that may have relevance to Cd transport is the ATP-binding cassette (ABC) transporter family. ABC proteins comprise the largest known protein family and have been found in a diverse range of organisms spanning all kingdoms. Most members of this superfamily are associated with membrane transport of a wide variety of substrates, including sugars, lipids, alkaloids, glutathione conjugates, and heavy metal chelators, among others (Theodoulou 2000). Because of the size of this superfamily as well as its clinical significance, this protein family has

generated considerable research interest. For instance, multidrug resistance (MDR) and multidrug resistance-associated (MRP) proteins have been implicated in the ability of cancer cells to simultaneously acquire resistance to structurally unrelated chemotherapeutic agents (Ishikawa et al. 1997; Theodoulou 2000). Structurally, members of this family generally contain at least one nucleotide binding fold (NBF) containing the ABC signature motif and two or three transmembrane domains (TMDs) (Theodoulou 2000; Sanchez-Fernandez et al. 2001). *Arabidopsis thaliana* has been found to contain 129 potential ABC proteins (Sanchez-Fernandez et al. 2001), which indicated the utility and importance of this family in plants.

Some ABC proteins have been shown to be involved with heavy metal transport. HMT1, isolated from *Schizosaccharomyces pombe*, was capable of transporting a low molecular weight (LMW) phytochelatin-Cd complex into isolated vacuoles (Ortiz et al. 1995). HMT1 was noted to be the first ABC-type transporter that conferred tolerance by mediating vacuolar sequestration of a toxic substance (Ortiz et al. 1995). Another vacuolar ABC protein, YCF1 from *Saccharomyces cerevisiae*, was found capable of transporting glutathione conjugates such as DNP-GS and metolachlor-GS and was also suggested to have a role in transporting toxic compounds into the vacuole (Li et al. 1996). Subsequent work with isolated vacuoles revealed that YCF was also capable of transporting bis(glutathionato)cadmium (Cd-GS_2) but not Cd-GS or any type of a Cd-phytochelatin complex (Li et al. 1997), which indicated different yeast species utilized different biochemical pathways for Cd detoxification. Although plants and the yeast *S. pombe* have been noted to share many similarities in their heavy metal detoxification pathways (Clemens and Simm 2003), no HMT1 homolog was found in the *Arabidopsis* genome, which suggested plants may use a different mechanism or different type of ABC transporter for Cd-PC sequestration in the vacuole (Sanchez-Fernandez et al. 2001). However, one ABC

transporter in *Arabidopsis*, ATM3, appeared to have a role in plant Cd tolerance since plants overexpressing this gene were more Cd tolerant and were able to accumulate more shoot Cd (Kim et al. 2006). Normally, ATM3 was noted to be involved in transport of Fe-S clusters from the mitochondria into the cytosol, which led the authors to suggest this transporter may be important for glutathione recycling.

Additionally, other transporters are likely to be involved in plant Cd transport and trafficking. Using gene chips, Wintz et al. (2003) examined the expression patterns of 8,300 *Arabidopsis* genes that experienced five days of Zn, Fe, or Cu deficiency. Although not all of the known or suspected metal transporting genes were present on the microarrays that were used, this work provided a useful tool for studying expression patterns of entire genetic systems in response to metal stress. Importantly, this research represented a first step towards understanding the coordinated expression patterns of different gene families involved in plant metal biochemistry and the complex coordination and control of the genetic system involved with plant metal transport. For instance, in addition to their roles as Zn transporters, AtZIP2 and AtZIP4 were found to be upregulated by Cu deficiency, although AtZIP4 required a much longer period of Cu deficiency before it showed a response (Wintz et al. 2003). Perhaps surprisingly, two members of the oligopeptide transporter family (OPT) were shown to be regulated by metal status, AtOPT2 and AtOPT3. The latter gene was expressed in yeast and appeared capable of Cu, Mn, and Fe transport (Wintz et al. 2003), which demonstrated the unexpected role of some genes in metal transport. These investigators also found evidence for metal regulation of ABC proteins, aquaporins, nicotianamine synthase genes, Fe reductases, and ferritins. Recently, a CATMA microarray capable of monitoring over 22,000 genes was used to study *A. thaliana* response to either 5 μM or 50 μM Cd concentrations over 2, 6, and 30 hour time periods (Herbette et al. 2006). Expression profiles were found to be more time-

regulated rather than dose-regulated and only a few common genes were identified across time points. Interestingly, gene response was more similar across concentrations at the same time point, which illustrated the time, rather than dose, dependency of the response in this experiment. Significant changes were noted in genes related to cell wall metabolism, signal transduction, oxidative stress, and sulfur metabolism (Herbette et al. 2006). These studies provided important insights in examining Cd response from a whole-network perspective, and appeared vital for future plant metabolic engineering efforts.

2.3.2. Energetics of transport

Transport of any substrate, including divalent cations, across the cell's plasma membrane is fundamentally governed by energetic considerations. Reid (2001) noted that the plant cell's cytoplasm has an overall negative potential and as a result an inwardly directed electrical gradient provided a major driving force for cation uptake from a plant's external environment. The magnitude of this gradient was stated to be around –200 mV (Clemens et al. 2002), and thus uptake of metal ions was suggested to be mainly through secondary transporters not directly utilizing ATP hydrolysis as an energy source. Another important consideration involved the activity ratio of the ion in question between the exterior of the plasma membrane and the cytoplasm, where it was noted that cytoplasmic ion activities were likely small due to ion complexation and precipitation. Although the possibility existed that this activity gradient could be outwardly directed, the strong inwardly directed electrical gradient was suggested to dominate and drive uptake in the case of cations (Reid 2001).

These fundamental energetic considerations must be taken into account when considering how the various classes of cellular transporters involved in metal uptake

and trafficking operate. A search of the literature showed, however, that energetic mechanisms were not well elucidated for many transporters and that while bioinformatics made it possible to search entire genomes and discover new classes of genes, detailed functional and mechanistic studies appeared to lag well behind. Sequence data has been helpful in elucidating the energetics of some transporter classes, though. For instance, genetic sequences of the P-type ATPases and ABC transporters have shown they contain ATP or nucleotide binding domains (Theodoulou 2000; Axelsen and Palmgren 2001). In the case of ABC transporters, direct evidence for ATP dependence has been shown for HMT1 (Ortiz et al. 1995) as well as for YCF1 (Li et al. 1997). As their name implied, energetics of the cation/H^+ antiporters were also well understood; AtCAX1 and AtCAX2 exhibited sequence similarity to known microbial Ca^{2+}/H^+ antiporters and experimental evidence from yeast established their dependency on a pH gradient (Hirschi et al. 1996).

Other classes of transporters appeared to be less well understood in terms of energetics, however. None of the plant NRAMP transporters were found to be mechanistically understood. Curie et al. (2000) proposed that the AtNRAMPs had ATP coupling subunits in the highly conserved consensus transport motif (CTM) region of the protein. However, Thomine and colleagues (2000) found that AtNRAMPs 3 and 4 exhibited pH dependent Fe uptake in yeast, which suggested a proton dependency of these transporters. These results agreed with the finding that a rat NRAMP member, DCT1, was a divalent metal/H^+ symporter under certain conditions (Gunshin et al. 1997). However, this same transporter exhibited a mechanistic H^+ 'slip' phenomenon that was influenced by the membrane potential and the presence of metal ions, which precluded a simple categorization (Nevo and Nelson 2006). Unfortunately, incisive studies demonstrating plant NRAMP energetics were not found. The energetics of ZIP proteins were also unclear. AtZIP2-mediated Zn

uptake in yeast was found to be pH dependent since no uptake was observed below a pH of 5, but AtZIPs 1 and 3 failed to display a similar pH dependence (Grotz et al. 1998). No other work on the energetics of these transporters was found.

Knowledge on CDF energetics was also incomplete; through expression in proteoliposomes loaded with NH_4Cl, AtMTP1 (ZAT) was found to not require a proton gradient for transport, and the Zn concentration gradient was suggested to provide the driving force (Bloss et al. 2002). In *R. metallidurans*, these same authors found evidence for $\Delta\Psi$ driven uniport. However, popular PtdMTP1 was only able to slightly rescue a Zn sensitive yeast phenotype when expressed in yeast defective in vacuolar H^+-ATPase activity, which suggested a proton gradient dependency (Blaudez et al. 2003). Additionally, ShMTP1, a CDF member from the acid tolerant tropical legume *Stylosanthes hamata*, was unable to rescue Mn sensitivity in yeast that were identically impaired in vacuolar H^+-ATPase activity, which also indicated a proton gradient was needed for transport (Delhaize et al. 2003). These authors suggested ShMTP1 had a Mn^{2+}/H^+ antiport function. CNGC transporters were yet another class of protein where an understanding of the energetics was incomplete. However, cyclic nucleotide binding has been found to be crucial for plant CGNC activity and activation through such binding was voltage insensitive or only weakly sensitive (Leng et al. 2002; Balague et al. 2003). The Cu transporting COPT family in *Arabidopsis* was found to be uncharacterized in terms of its energy dependencies, although no ATP binding domain was reported in the sequence and hence it may be a secondary transporter of some kind. Finally, the energetics of MATE transporters were also not completely understood. Preliminary work showed CCCP inhibition of AtDTX1 transport of norfloxacin, which suggested dependency on the proton motive force but the exact mechanisms remained unclear and required further study (Li et al. 2002). Additionally, *Xenopus* oocytes that expressed FRD3-GFP fusion proteins were noted

to have unaltered citrate transport properties when the pH or Na concentration of the external bathing solution was altered, which appeared to indicate that citrate transport by FRD3 did not rely on a pH or Na gradient (Durrett et al. 2007). Clearly, many transporters involved in heavy metal transport in plants were not fully characterized and additional research remained.

2.3.3. Plant heavy metal responses

Cd is a heavy metal for which no physiological function has been found in plants. Due to this, plants have no known nutritional requirement for Cd; indeed, exposure to it has been shown to be toxic and damaging to plant cells. Thus, plants have had to devise various ways through which exposure to toxic compounds can be mitigated or avoided. The sessile nature of plants means that they necessarily have a sophisticated cellular transport and sequestration network since they are required to derive all their needed chemicals—and deal with any problematic ones—in the local environment where they live. This, in turn, helps explain why so many transporters for cations and other substrates have been found in the *Arabidopsis* genome (Maser et al. 2001; Sanchez-Fernandez et al. 2001). In addition to sophisticated transporter systems, though, research has found that plants also use other processes, such as chelation and sequestration, when confronted with heavy metals. These processes and the general effects of Cd on plants are reviewed here.

2.3.3.1. General Cd effects

Cd has been suggested to have a wide range of effects on plants. Di Toppi et al. (1999) noted chlorosis, altered root RNA synthesis and ribonuclease activity,

disruption of nitrate biochemical pathways, inhibition of Fe reductase activity, altered water balance, and inhibition of stomatal opening all as general effects of Cd. Good "target sites" for toxicity were suggested to be molecules that relied upon the presence or binding of Zn or Ca for proper activity (Clemens 2006b). Additionally, this heavy metal was implicated in affecting the activity of many plant enzymes such as rubisco, glucose-6-phosphate dehydrogenase, carbonic anhydrase, and glutamate dehydrogenase, among others (Prasad 1995; di Toppi and Gabbrielli 1999). Di Toppi and Gabbrielli (1999) also noted that Cd exposure produced oxidative stress and decreased activity of antioxidant enzymes such as superoxide dismutase and catalase. According to these authors, Cd exposed plants have additionally been found to synthesize heat shock or stress proteins with widely varying molecular masses, which were possibly upregulated due to Cd induced misfolding of proteins. Notably, Cd is not redox-reactive, and the observed oxidative stress was likely due to GSH depletion caused by formation of phytochelatins and through direct binding of Cd to GSH (Clemens 2006b). Prasad (1995) stated that Cd inhibited net photosynthesis in a variety of plants, including *Phaseolus vulgaris*, *Zea mays*, and barley, generally through an inhibition of PS II. However, the exact mechanism through which Cd disrupted photosynthesis was not clear; stomatal closure, photophosphorylation inhibition, and disruption of the Calvin cycle were possible mechanisms (Prasad 1995).

Schutzendubel and Polle (2002) went on to further elucidate some of the various effects of heavy metals on plants, and focused particularly on how plants cope with oxidative damage. Noting the high affinities of heavy metals for sulfur and their ability to bind cysteine residues in proteins, three general mechanisms of metal toxicity were proposed: (1) production of reactive oxygen species, (2) displacement of metal ions from molecules, and (3) blocking or binding to important functional groups

in molecules (Schutzendubel and Polle 2002). In general, Cd was said to result in diminished antioxidative defenses and a subsequent plant stress response. Using metabolic modeling of a plant's oxidant scavenging ability, these authors ran computer simulations and suggested that Cd exposure resulted in highly elevated H_2O_2 levels. Thus, a general response model was proposed where a reduced antioxidative capacity resulted in higher plant H_2O_2 levels which in turn acted as signals for secondary defenses. These secondary defenses would cause cell wall rigidification and lignification, which was proposed to ultimately lead to cell death (Schutzendubel and Polle 2002). Owing to between-species variability, there didn't appear identical responses to Cd among plants from the studies reviewed here, but the heavy metal was clearly able to exert multiple damaging effects on plant cells.

2.3.3.2. Phytochelatins

Although Cd has been shown to be disruptive to a plant's biochemistry, many plants have been found to exhibit certain protective responses. Chief among these responses was the induction of phytochelatins (PCs) in response to heavy metal stress. Phytochelatins were first isolated from plant cell suspension cultures and found to have the general structure $(\gamma\text{-glu-cys})_n\text{-gly}$ (Grill et al. 1985). These molecules were found in a wide range of plants and were capable of being induced by multiple heavy metals, although at high concentrations (Grill et al. 1987). Due to the γ-glu linkage and the finding that buthionine sulfoximine, a γ-glutamylcysteine synthetase inhibitor (a crucial enzyme in glutathione biosynthesis), was able to prevent PC formation, PCs were suggested to be non-translationally synthesized from glutathione (Grill et al. 1987). Notably, in some plants with a glutathione homolog where the C-terminal

amino acid was altered, such as with ser, -β-ala, or glu, correspondingly altered PCs have been found and termed *iso*-PCs (Zenk 1996).

The enzyme responsible for this synthesis, γ-glutamylcysteine dipeptidyl transpeptidase (also known as phytochelatin synthase), was later isolated and purified from suspension cultures of *Silene cucubalus* (Grill et al. 1989). The enzyme was suggested to be a tetramer with four M_r 25,000 subunits, and required the presence of metal ions for activity in catalyzing the following basic reaction (Grill et al. 1989):

$$(\gamma\text{-glu-cys})_n\text{-gly} + \gamma\text{-glu-cys-gly} = (\gamma\text{-glu-cys})_{n+1} + \text{gly} \qquad \text{[Eq. 2.4]}$$

Interestingly, the presence of glutathione was not required for formation of hepta- and nonapeptide chains from a pentapeptide substrate, which indicated the ability of pentapeptides to act simultaneously as both donors and acceptors of γ-glu-cys peptides (Grill et al. 1989). Aside from the production of metal binding phytochelatins, PC synthase appeared to have another important cellular function in the metabolism of glutathione-S conjugates in plants (Beck et al. 2003). These investigators found that AtPCS1 catalyzed the formation of bimane-EC from bimane-GS; in essence, the enzyme catalyzed the rate limiting step of cleaving the C-terminal glycine residue from xenobiotics conjugated to GSH. Other GSH-conjugated substrates, such as acetamidofluorescein, uracil, benzyl, phenylbenzyl, and nitrobenzyl also underwent the same reaction in the presence of AtPCS1 (Beck et al. 2003). Interestingly, like PC formation, recombinant AtPCS1 required the presence of metals for GS-conjugate carboxypeptidase activity.

Despite purification of phytochelatin synthase and elucidation of its basic mechanism, another decade was needed before the gene encoding this protein was concurrently isolated by three groups (Clemens et al. 1999; Ha et al. 1999; Vatamaniuk et al. 1999). The protein was found to localize primarily in the cytosol (Vatamaniuk et al. 1999) and did not require the presence of a vacuolar proton

gradient or even vacuolar structures to confer increased Cd tolerance to yeast (Clemens et al. 1999). As was expected, the gene was constitutively expressed in *Arabidopsis* (Ha et al. 1999) and wheat (Clemens et al. 1999), but Clemens et al. (1999) found evidence for enhanced gene expression under Cd stress while Ha et al. (1999) did not. Therefore, the gene may have been regulated differently in different species or the methods used to quantify expression were too variable to reveal consistent trends. Prior work (Grill et al. 1989) had suggested constitutive expression and little, if any, responsiveness to metal levels. With the genetic code for PC synthase revealed, database searches quickly revealed a homolog in the nematode *Caenorhabditis elegans* (Ha et al. 1999; Vatamaniuk et al. 1999), which indicated that PCs may have roles in members outside of the plant kingdom. More recently, PCS homologous sequences were noted to occur in representatives across all eukaryotic kingdoms (Clemens 2006a). However, the activity of many of these genes awaited experimental confirmation, and thus the overall importance of PCS in other kingdoms was unknown.

The discovery of the PC synthase gene has also called the basic mechanisms of the enzyme into question. Grill et al. (1989) originally proposed the metal dependent transpeptidation reaction that was described previously. In one model, the enzyme was suggested to contain metal binding residues in both the N- and C-termini; however, N-terminal residues were absolutely required for metal binding and enzyme activity while the C-terminal residues, upon binding, brought the metal ions in contact with the catalytic N-terminal (Cobbett 1999; Cobbett 2000). Thus, this model proposed direct binding of metal ions to the enzyme. Another group (Vatamaniuk et al. 2000) proposed a model where PC synthase was activated by a Cd-GS_2 complex rather than free metal ions. In their model, Cd-GS_2 and GSH served as co-substrates for the enzyme that catalyzed a "ping-pong" two stage reaction:

$$\text{AtPCS1} + \gamma\text{-Glu-Cys-Gly} \rightarrow \text{AtPCS1-}\gamma\text{-Glu-Cys} + \text{Gly} \qquad \text{[Eq. 2.5]}$$

$$\text{AtPCS1-}\gamma\text{-Glu-Cys} + \text{Cd}\cdot(\gamma\text{-Glu-Cys-Gly})_2 \rightarrow$$

$$\text{AtPCS1} + \text{Cd}\cdot(\gamma\text{-Glu-Cys})_2\text{-Gly} + \gamma\text{-Glu-Cys-Gly} \qquad \text{[Eq. 2.6]}$$

In support of this mechanism, the authors performed equilibrium calculations which showed that the free Cd concentration in their reaction media hovered around 10^{-13} M while the enzyme binding constant (K_L) for Cd was $0.54 \pm 0.21 \times 10^{-6}$ M. In addition, blocked thiols such as S-methylglutathione were able to catalyze the formation of S-methyl-PCs in the complete absence of heavy metals (Vatamaniuk et al. 2000), which appeared to further support the idea that free metals were not required for enzyme activity. However, results from this study were strongly disputed by Oven et al. (2002). In a reaction mixture with both Cd-GS_2 and S-methyl-GSH, they calculated the amounts of $-SCH_3$ and –SH groups that were transferred by the enzyme. When Cd-GS_2 was present in very low amounts they found that nearly equal amounts of $-SCH_3$ and –SH groups were transferred, but as Cd-GS_2 concentration rose, the ratio of SCH_3:SH groups increased. This result was the opposite of what would be expected if Cd-GS_2 were being used as a substrate (Oven et al. 2002). These authors also noted that the type of buffer used had an effect on enzyme activity; formation of S-methyl PCs was shown to be significantly higher in HEPES-BTP buffer than in Tris-HCl buffer. However, Cd was previously shown in a NMR study to form stable complexes with Tris buffer, but not MES, HEPES, and MOPS buffers (Girault et al. 1998), and thus the use of Tris-HCl by Oven et al. (2002) likely impacted the free Cd in solution which in turn resulted in altered enzyme rates due to the requirement of some form of Cd binding for enzyme activity. Additionally, the presence of thiols, such as DTT, β-mercaptoethanol, and L-cysteine, was shown to be adequate for enzyme catalyzed transfer of SCH_3 groups—i.e. production of S-methyl

PCs (Oven et al. 2002). However, all reactions used Cd or Zn concentrations of 0.5 mM, which may have been enough to cause enzyme poisoning and cloud results. Beck et al. (2003) suggested that results from Vatamaniuk et al. (2000) may have been confounded since reactions were not performed in a reducing environment and thus the enzyme was partially oxidized, which may have influenced metal binding measurements and enzyme activity.

Further debate existed over activation of PC synthase. Maier and colleagues (2003) created two immobilized peptide libraries of 13-mers using PC synthase from *Schizosaccharomyces pombe* and *Triticum aestivum*. These libraries were used in metal binding assays with radiolabeled 10 μM $CdCl_2$ which revealed six and seven Cd binding sites in these two enzymes, respectively. In general, the binding sites contained highly conserved cysteine residues among the known PC synthase family members as well as the presence of charged residue groups (Maier et al. 2003b). Additionally, Cd binding was found to be reduced, but not abolished, in the presence of 1 mM GSH while a 5-fold excess of Cu was sufficient to abolish Cd binding at some sites. Further site directed mutagenesis of AtPCS1 (Maier et al. 2003b) demonstrated that Cys-56, Cys-90, Cys-109, and Cys-113 mutants all failed to restore Cd tolerance when transformed into *Δpcs S. pombe* strains, although Cys-56 appeared to result in the strongest growth inhibition while slightly less severe effects were evident in the other mutants. These cysteines corresponded to binding site residues in the TaPCS1 sequence, which implied direct metal binding was critical for activation, catalysis, or both. A later report by Vatamaniuk et al. (2004) provided direct evidence for establishment of phytochelatin synthase as a dipeptidyl transferase that has two distinct acylation sites. One of the sites did not require metals to be present for GSH binding while the other site appeared to exhibit Cd dependent binding and concomitant PC production. Furthermore, through site-directed mutagenesis, Cys-56

was found to be critical for acylation of the metal-independent primary site as well as basic PC production (Vatamaniuk et al. 2004), which supported the previously established importance of this residue as reported by Maier et al. (2003), despite disagreement over the theoretical underpinnings of this importance. Other mutagenesis results, however, differed sharply from those of Maier et al. (2003) in that Cys-56 was the only residue that appeared critical for the growth of a ycf1Δ mutant strain of *Saccharomyces cerevisiae* while other mutated residues (Cys-90, Cys-91, Cys-109, and Cys-113) did not result in reduced growth. Reasons for these differences in mutagenesis results were not immediately clear, although differences between recombinant techniques and yeast strains may have aided in clouding results. Unfortunately, the second proposed acylation site, the one that presumably needed metal or a Cd-GS_2 complex for activation, was not identified, although it was suggested to contain a Thr or Tyr residue capable of forming oxyanions (Vatamaniuk et al. 2004). Thus, the debate over how PC synthase underwent activation was unresolved and additional research is clearly needed.

2.3.3.3. Location of PCs

Phytochelatins have generally been found throughout all parts of the plant. In studying the effects of long term Cd exposure on *Brassica napus* grown in soil artificially contaminated with varying Cd levels (10-200 mg Cd/kg soil, weight type not reported), a sharp decrease in PC leaf levels was found as plants aged (Carrier et al. 2003). Interestingly, leaf Cd concentration also declined with time, from approximately 250 mg/kg-DW to about 150 mg/kg-DW between 20 and 69 days after sowing, respectively. Leaf biomass increased over this time, notably during the latter parts of the trial, and thus *B. napus* evidently overcame initial inhibition by Cd and

PCs appeared to be involved mainly in the initial acute stages of Cd toxicity (Carrier et al. 2003). Acute responses to high metal levels, however, do not appear to be the only role of PCs *in vivo*; environmental Cd levels (pCd = 9.6) in a flow-through hydroponic system resulted in enhanced phytochelatin levels in romaine lettuce compared to controls (Maier et al. 2003a).

After synthesis in response to metal stress, PCs may have roles in long distance metal transport in plants. Using the cad1-3 mutant of *Arabidopsis*, TaPCS1 from wheat was transformed into this strain under the control of the alcohol dehydrogenase promoter (Adh::TaPCS1) which limited gene expression to root tissue (Gong et al. 2003). The root-specific expression was confirmed by RT-PCR as well as mRNA and protein blots, and the transgenic plants did not display the metal-sensitive phenotype of cad1-3 plants. Interestingly, PCs were detected in the stem and leaf tissue of plants expressing Adh::TaPCS1 despite the absence of PCS protein in these tissues, which provided evidence for long distance root-to-shoot transport of PCs (Gong et al. 2003). Although overall PC levels were similar between WT controls and plants that expressed TaPCS1, transgenic lines contained more Cd in their stem and leaf tissue. The mechanism behind this phenomenon, however, was unclear. Additional work by this same laboratory found evidence for long distance shoot-to-root transport of PCs in *Arabidopsis* as well (Chen et al. 2006). Here, TaPCS1 was expressed in cad1-3 plants under the control of the shoot-specific CAB1 promoter, which was confirmed by northern blot and mRNA analysis. Although the transgenic plants were unable to rescue the root-sensitivity of cad1-3, PC_2 was clearly detected in root tissue by HPLC and mass spectrometry. Furthermore, grafting of a WT shoot onto a cad1-3 atpcs2-1 (plants were lacking both the PCS1 and PCS2 genes) resulted in detection of PC_2, PC_3, and PC_4 in roots, which demonstrated that under their native promoter, PCs were also capable of shoot-to-root transport (Chen et al. 2006). Taken together, these papers

provided strong, although indirect, evidence for long distance PC transport in plants, and therefore a possible involvement of PCs in long distance metal transport.

PCs were also suggested to have roles in plant metal homeostasis, such as with Zn and Cu, which was based upon the observation of PC induction when plant cell suspension cultures were transferred from Zn and Cu deficient media to new media replete with these ions (Zenk 1996). In agreement with this hypothesis, control lettuce plants (with no Cd exposure) grown in a nutrient solution had detectable levels of PCs (Maier et al. 2003a). Although PCs may have metal trafficking functions, their general role in Cd biochemistry was noted to be sequestration and subsequent transport to the vacuole, where the PC may be broken down and partially recycled (Zenk 1996). Some plants have been noted to form high molecular weight (HMW) complexes that consisted of Cd-S crystals coated with PCs which enhanced Cd sequestering ability and stability of the complex (Cobbett 2000). Taken together, PCs may have roles in detoxification, long distance metal transport, and metal homeostasis. Additional experiments are needed to further verify these roles and ascertain if such roles are universal or variable among different species.

2.3.3.4. Plant metallothioneins and other ligands

In addition to phytochelatins, plants have been noted to use other ligands that are involved with heavy metal biochemistry. Metallothioneins (MTs) were one such ligand; they are low molecular weight cysteine-rich metal binding proteins (for reviews, see Clemens 2001, Rauser 1999) that have been found in both plants and animals. These proteins were divided into two classes: (1) class I MTs which were found only in animals and include the well studied mammalian MTs that were implicated in Cd detoxification and metal homeostasis, and (2) class II MTs which

were designated as such due to divergent arrangements of cysteine residues (Clemens 2001). Only the second class of MTs were reported in plants, although class II MTs were found in invertebrate animals and unicellular organisms as well (Rauser 1999). In general, plant MTs were found to have two or three cysteine rich metal binding regions separated by a variable length linker region and were divided into types 1-4 based upon organization of the cysteine residues (Rauser 1999).

Despite the well established presence of MTs in plants, the physiological function of these small proteins appeared less clear. Cu tolerance among tolerant and non-tolerant ecotypes of *Silene vulgaris* was studied and was associated with higher constitutive expression of SvMT2b, a class II MT gene (van Hoof et al. 2001). However, by studying gene segregations results from crosses between tolerant and non-tolerant parents, the authors concluded that SvMT2b overexpression, by itself, was not sufficient to confer Cu tolerance although the highly Cu tolerant lines had high expression of this gene (van Hoof et al. 2001). Interestingly, these authors found that a Cd sensitive yeast which was transformed with this gene was able to grow at 10-fold higher Cd levels (0.1 mM $CdSO_4$ versus 0.01 mM $CdSO_4$) which demonstrated the ability to confer Cd tolerance, but whether the gene had a role in Cd tolerance *in vivo* was not investigated. In general, though, Cd binding in plants has been largely attributed to PCs. A study that examined expression of five *Arabidopsis* MT genes by using MT:GUS fusion proteins found tissue specific expression patterns that tended to increase in intensity as plants aged (Guo et al. 2003). Furthermore, the effects of a two day Cu treatment on MT gene expression were investigated. The authors noted that while many tissues did not exhibit altered expression levels, several did show higher expression levels of one or more MT genes. These included young expanding leaves, root tips, phloem and vascular tissue in mature leaves, mature roots, and trichomes, which were unique in that all MT:GUS fusions showed enhanced

expression. Increased expression of all but one MT gene was observed during senescence events as well. In general, the MTs studied were attributed metal homeostasis roles of varying degrees: some for housekeeping, some for rapid changes in Cu levels, and some for Cu tolerance (Guo et al. 2003). This conclusion agreed well with a review (Clemens 2001) that suggested MTs have roles in metal detoxification, Zn buffering, metal scavenging during senescence, and metal secretion into leaf trichomes. However, no definitive role for MTs in plant Cd biochemistry was found.

In addition to MTs and PCs, other ligands have been suggested to be involved in metal binding and may be particularly important to long distance transport of Cd in the plant. Small organic acids, including amino acids, may have roles in Cd binding, primarily through chelation. In a review (Rauser 1999), citrate, malate, and oxylate were all suggested to have possible roles in Zn chelation, although there appeared to be variable results that caused a lack of agreement on which, if any, organic acid played a dominant role. Rauser (1999) also noted that when vacuolar conditions were simulated using GEOCHEM-PC, a computer speciation program, the same three acids were predicted to complex the majority of Cd, although the review did not note if the contribution of PCs or other sulfur containing complexes were included in the calculations. Another review (Clemens et al. 2002) suggested that chelator type was the primary determinant of metal destination within the cell: sulfur containing ligands such as PCs and MTs could route metals into root vacuoles while other organic acids and compounds such as histidine, nicotianamine, and citrate may direct metals to the xylem and thus facilitate above ground transport. One of the few studies that looked at Cd ligands *in vivo* used X-ray absorption spectroscopy (XAS) to determine Cd binding in roots, shoots, and leaves of *Brassica juncea* (Salt et al. 1995b). In roots and shoots, Cd-S bonds with four ligands were prevalent while xylem sap data suggested

Cd coordination with six oxygen or nitrogen ligands (Salt et al. 1995b). Therefore, PCs did not appear to play a role in xylem transport of Cd, while ligand switching occurred when Cd was sequestered in the shoot. The mechanisms through which such a ligand switch would occur were unknown. In a review of their work (Salt et al. 2002), it was suggested that translocated Cd resulted from a couple of possibilities: either it never underwent sulfur coordination and was never stored, or it was moved from storage to transport forms through an active process. These results, though, were at odds with PC transport studies in *Arabidopsis* that were previously detailed in this review (Gong et al. 2003; Chen et al. 2006), as well as a study that directly detected PCs in the xylem sap of *B. juncea* in response to Cd stress (Wei et al. 2007). However, the possibility existed that the XAS experiments on xylem sap were contaminated with atmospheric oxygen and nitrogen, which would have skewed results (Ahner, personal communication). No further evidence was found to suggest which, or either, of these possibilities was more tenable.

2.3.4. Hyperaccumulator Cd biochemistry

As stated previously in this review, hyperaccumulator plants have the ability to accumulate substantial concentrations of metals in above-ground plant tissue. Therefore, these plants may have roles in phytoextraction or may serve as roadmaps guiding the development of high biomass hyperaccumulators. However, the exact genetic and molecular basis through which these plants are able to accumulate and prosper under normally toxic tissue metal loads was not well understood in the literature. A review (Assuncao et al. 2003b) summarized the state of knowledge about hyperaccumulator heavy metal biochemistry and suggested the use of *T. caerulescens* as a model species to study metal accumulation traits. In the review presented here,

findings relevant to Cd hyperaccumulation as well as some interesting research questions are presented. In particular, metal uptake, trafficking, and chelation are discussed along with a brief overview of topics concerning hyperaccumulator evolution.

Enhanced metal uptake was one way hyperaccumulators were found to increase their metal content. For instance, Zn uptake into the roots of *T. caerulescens* was found to occur faster than in roots of *Thlaspi arvense*, which translated to a factor of 4.5 higher V_{max} (0.270 μmole Zn/g-h versus 0.060 μmole Zn/g-h) value for the hyperaccumulator (Lasat et al. 2000). These transport properties were found to be associated with a high constitutive mRNA level of TcZNT1, a ZIP family member, when *T. caerulescens* was grown in the presence of 1 μM Zn (Pence et al. 2000). Additionally, yeast that expressed TcZNT1 were capable of Cd transport at rates similar to that of Zn (Pence et al. 2000), although plant Cd uptake was not investigated which made it impossible to discern if TcZNT1 was involved with Cd transport *in vivo*. Also, no TcZNT1 knock out plant lines were created, and thus the absolute importance of this gene to Zn and/or Cd hyperaccumulation was not completely established.

Enhanced expression of this gene was not the only factor that may have explained high Cd uptake observed in *T. caerulescens*. Lombi et al. (2001) noted differences in Cd accumulation between various *T. caerulescens* ecotypes and the peculiar ability of the Ganges ecotype from southern France to accumulate Cd. They compared Cd and Zn uptake in Ganges to another ecotype, Prayon, that also accumulated Zn and Cd. Ganges plants were found to exhibit a much higher V_{max} (160 nmol/g-FW-h versus 33 nmol/g-FW-h) than those from Prayon when placed in Cd solutions (Lombi et al. 2001b). Zn uptake characteristics appeared similar for both ecotypes, but in a solution with equimolar amounts (10 μM) of Cd and Zn, the Ganges

ecotype did not show reduced root Cd uptake while a 33% reduction was observed in Prayon (Lombi et al. 2001b). Therefore, Ganges plants appeared to have a transporter capable of high affinity Cd transport that was not present in Prayon plants, although no molecular evidence in support of this hypothesis was presented. Using a depletion technique, further kinetics experiments revealed that separate additions of La, Verapamil, Ca, Zn, Co, Cu, Mn, Ni, and Fe(II) to equimolar amounts of Cd (5 μM) were unable to yield a statistically significant effect on Ganges Cd uptake while some of these treatments reduced Cd uptake in Prayon plants (Zhao et al. 2002). Additionally, Fe deficiency was shown to substantially increase V_{max} in Ganges plants from 62.6 to 187.6 nmol/g root FW-h (a factor of 3) while it did not result in an appreciable increase in Prayon V_{max} (Lombi et al. 2002). These authors also cloned a gene from Ganges, TcIRT1-G, a Fe transporter belonging to the ZIP family of metal transporters. Northern blots showed TcIRT1-G mRNA was strongly up-regulated under –Fe conditions in Ganges, but was barely detectable (using Ganges probes) in Prayon plants under –Fe stress (Lombi et al. 2002). They concluded that TcIRT1-G was responsible for enhanced Cd uptake under Fe deficient conditions but stated that enhanced Cd uptake by Ganges under Fe sufficient conditions was unexplained. However, given that a solution containing 5 μM Cd and 5 μM Fe failed to reduce Ganges uptake appreciably compared to a solution lacking Fe (Zhao et al. 2002), the TcIRT1-G transporter appeared to display a preference for Cd over its "native" Fe substrate, which may partly explain the persistence of high Cd uptake in Ganges under Fe sufficient conditions. Although TcIRT1-G mRNA was barely detected, if at all, in Fe replete plants, the possibility existed that it was still present in low amounts facilitating Cd transport; alternatively, another transporter could have been responsible for the enhanced Cd uptake observed in Ganges plants grown in full strength media.

In addition to enhanced transport into roots, hyperaccumulators have been found to display altered intracellular metal trafficking properties. In particular, hyperaccumulating plants usually had shoot-to-root concentration ratios of one or greater and generally appeared to use their shoots, rather than roots, as final storage spaces for heavy metals. In a review, Lasat et al. (2000) noted enhanced shoot Zn accumulation in *T. caerulescens* when compared to *T. arvense*. Compartmental analysis using radiolabeled Zn of vacuolar, cytoplasmic, and cell wall fractions suggested that root vacuoles of *T. caerulescens* effluxed Zn at approximately twice the rate of *T. arvense* (Lasat et al. 1998). However, efflux studies of this type with divalent cations may have suffered from methodological problems (Spanswick, personal communication) and since no comparative efflux study with dead roots was performed, results may not be conclusive or quantitative. Additionally, at external Zn concentrations of 1000 μM which was suggested to be representative of typical xylem sap concentrations in the hyperaccumulator, faster metal uptake was observed in leaf tissue from *T. caerulescens*, although no distinct transporter was identified and isolated leaf protoplasts from the two species showed no statistically significant difference in uptake (Lasat et al. 1998). In *A. halleri*, growth in hydroponic media containing elevated levels of Zn and Cd resulted in plants that had high metal concentrations in the base of leaf trichomes as well as in certain mesophyll cells (Kupper et al. 2000). The trichomes contained extraordinarily high metal levels that could exceed 1 M; EXDA analysis showed P- and S- ligands were not involved in this compartment and the preferential storage in certain mesophyll cells was suggested to indicate the importance of these cells as a sink in later stages of hyperaccumulation (Kupper et al. 2000).

Root and leaf heavy metal responses were not the only alterations found in hyperaccumulator behavior. As expected of plants that allocate large amounts of

metal to their above-ground biomass, vascular tissue played a crucial role, along with possible ligand binding, in this long distance transport. In this regard, no specific study was found that exclusively focused on Cd ligands in hyperaccumulators, although results from some studies using Zn were instructive. Lasat et al. (1998) found significantly more Zn in the xylem sap of *T. caerulescens* than *T. arvense*. They examined organic and amino acid content of the sap and found that acetate was the only organic acid that showed any response to increasing Zn concentrations; they failed to detect aconitate, citrate, fumarate, pyruvate, and succinate in either *T. caerulescens* or *T. arvense*. Additionally, amino acid contents of the xylem sap from the two plants were similar; the only notable differences were a higher Glu content as well as the complete absence of His in *T. caerulescens* (Lasat et al. 1998). A pair of studies used EXAFS to study metal ligands in tissues of *T. caerulescens* (Salt et al. 1999) or *A. halleri* (Sarret et al. 2002). In *Thlaspi*, results suggested root Zn was complexed with histidine while Zn found in the xylem was mainly in the form of a free hydrated cation with a small amount complexed with citrate (Salt et al. 1999). In the shoot, their results suggested complexation with citrate (38%), histidine (16%), cell wall (12%), and oxalate (9%), while the remaining Zn appeared to be present as a free hydrated ion. Sarret et al. (2002) compared the accumulator *A. halleri* to *Arabidopsis lyrata* and the effects of hydroponic growth versus natural growth of *A. halleri* at an industrially contaminated site. Their results differed from Salt et al. (1999) in that Sarret et al. (2002) reported that soil grown plants had citrate, phosphate, and malate forms of Zn in the roots and predominately malate-bound forms of Zn in the leaves. However, trichomes were found to have high Zn concentrations at the base and the chemical form of Zn was unknown in these structures, although coordination to an organic acid was postulated (Sarret et al. 2002). The major difference between hydroponic and industrial site growth was in the form of Zn found

in the roots: *A. halleri* grown in hydroponics had Zn phosphate as the major form of this metal in the roots, which was suggested to be the result of precipitation on the root surface (Sarret et al. 2002). Thus, there appeared to be differences among the way different species of hyperaccumulators may sequester the same metal; even experiments performed on the same species did not yield identical results, although methodological differences may have contributed to these disparities.

Since hyperaccumulators appeared to have numerous changes in their metal management strategies, microarray experiments focused on large fractions (or even the entirety) of a plant's transcriptome provided a promising systematic and comparative way of examining changes between normal and hyperaccumulating plants. One such study compared the transcriptome responses of *A. thaliana* and the hyperaccumulator *A. halleri* to a 50 μM or 10 μM Cd treatment along with a separate 10 μM Cu treatment (Weber et al. 2006). In order to achieve similar cellular Cd levels across species, approximately 2.5-fold more Cd had to be applied to roots of *A. halleri* over a two-hour period, which indicated reduced basal Cd uptake, at least in short term experiments. Interestingly, the number of Cd-responsive genes relative to the number of transcripts was 10-fold lower in *A. halleri* than in *A. thaliana*. Only five genes were found to be up-regulated in response to Cd in *A. halleri* while 111 were up-regulated in *A. thaliana*; four of these genes were shared in common by both plants. Additionally, five *A. thaliana* metal-responsive genes were found to be constitutively highly expressed in *A. halleri*. However, when eliminating genes that also showed a response to Cu, only 2 and 23 genes were specifically responsive to only Cd in *A. halleri* and *A. thaliana*, respectively. Taken together, the following conclusions were reached by the authors (Weber et al. 2006): (1) little evidence for short term, Cd-induced, oxidative stress was found, but an apparent Zn deficiency and high number of misfolded proteins was noted; (2) *A. halleri* Cd tolerance was partly due to lower short

term Cd uptake rates and more efficient Cd sequestration; (3) constitutive high expression of some metal-responsive genes in *A. thaliana* may have helped in the adaptation of *A. halleri* to metal-rich soil; (4) distinct signaling cascades exist for Cd ions; and (5) comparative transcriptome analysis by cross-species hybridization is a robust technique. Although this work provided numerous useful insights, only approximately 8000 genes were analyzed on the gene chips, and it remained possible that a larger array may have provided for more comprehensive, or even completely different, conclusions on the Cd responses of these two species.

Clearly, many of the mechanisms hyperaccumulators used to acquire high amounts of metals and translocate them to above-ground biomass while avoiding toxicity were not well understood. One of the major metal detoxification peptides in non-accumulators, the phytochelatins, have been suggested to have minor or reduced roles in hyperaccumulator metal tolerance. Ebbs et al. (2002) studied PC synthesis in the Prayon ecotype of *T. caerulescens* and compared it to *T. arvense* under identical experimental conditions. Interestingly, shoot PC levels were generally similar between the two species along the concentration range tested (0-50 μM) but yet *T. caerulescens* accumulated more Cd in the shoot, while root PC levels of the hyperaccumulator were significantly lower than those of *T. arvense* at Cd concentrations > 25 μM. These results pointed against a role for PCs providing tolerance to *T. caerulescens* although the possibility remained that higher efficiency use of PCs—such as in HMW complexes—by the hyperaccumulator could have been a viable mechanism (Ebbs et al. 2002).

Other work performed by Schat et al. (2002) studied PC response to metal stress in several species that included metal-resistant and non-resistant types in an attempt to elucidate the different roles PCs may have in metal adapted plants. In *Silene vulgaris*, Cd induced PC formation in both tolerant and non tolerant ecotypes,

but much higher PC levels were found in the non-tolerant ecotype at all Cd levels. Treatment with buthionine sulfoximine (BSO), an inhibitor of γ-ECS, which is a crucial enzyme in the GSH biosynthetic pathway, dramatically inhibited root growth in non-tolerant plants while the tolerant *S. vulgaris* ecotype showed no signs of root growth inhibition (Schat et al. 2002). In the three *T. caerulescens* ecotypes that were studied, Cd was noted to induce PC synthesis in amounts that were inversely proportional to the ecotype's relative sensitivity to Cd—i.e. the higher the degree of Cd resistance, the lower the degree of PC induction that was observed. BSO treatment failed to inhibit root growth in any of the three *T. caerulescens* types, but severe toxicity was observed in the shoots of the serpentine (Cd sensitive) ecotype. The tolerant ecotype displayed reduced shoot growth at 125 μM Cd, although the authors noted that chlorosis was apparent at 25 and 125 μM, in both the +BSO and –BSO treatments. Overall, their results suggested that high Cd tolerance was not dependent upon high PC levels, but again the issue of PC usage and efficiency was left unaddressed. Until more definitive mechanisms that govern the hyperaccumulating phenotype are discovered, PCs cannot be excluded from having a role in hyperaccumulator tolerance. Gene knockout experiments on PCS in these plants could potentially provide valuable insights, but no reports detailing these types of experiments were found. In addition, no studies involving MTs and their role(s) in hyperaccumulators were found.

2.4. Depletion measurement systems and Cd uptake kinetics

2.4.1. Hydroponics based depletion measurement systems

Hydroponic systems have been frequently used by researchers to grow plants in laboratory settings. The use of hydroponics has offered several advantages for research purposes and has been of particular use in studies of plant mineral nutrition since precise control of nutrient activity can be maintained. Additionally, various confounding factors associated with soil-based plant growth such as soil temperature, pH, water supply to the roots, nutrient desorption from the soil matrix and subsequent mass transfer—through convection or diffusion—to the root surface have been avoided or better managed in hydroponic systems (Wild et al. 1987). The following model was used to describe nutrient transfer from the soil to the plant (Clement et al. 1974):

$$M(solid) \leftrightarrow M(solution) \leftrightarrow M(plant\ roots) \leftrightarrow M(shoot) \qquad \text{[Eq. 2.7]}$$

Therefore, use of hydroponic systems eliminates the solid-to-solution reaction and focuses the research on measuring biological nutrient demands by the plant.

These types of systems have also been used to quantify nutrient depletion in the solution as a function of time and hence derive root uptake rates. Both cation and anion uptake rates were measured through the use of flowing culture systems, although their design, purpose, and use differed greatly. In a review, two fundamentally different types of systems were recognized: (1) nonrecirculating systems where nutrient solution flowed to waste and (2) recirculating systems where solution was reused (Wild et al. 1987). Other design differences included variation in control and instrumentation complexity, measurement techniques, temporal resolution, automated features, and feedback functions. Clement et al. (1974) designed a large,

complex system for measuring ion depletion through use of ion selective electrodes (ISEs), which was later referred to as the "Hurley System" (Wild et al. 1987). Their design used large tanks containing 300 L of solution for long term growth of plants and measured nutrient uptake through volumetric depletion of known replenishment stock solutions. Initially, their system measured nitrate consumption. Another complex system, called the Phyto-Nutri-Tron (Lorenzen et al. 1998), offered separate control of the root and shoot environments. This system used 16 growth cabinets arranged in two groups with each cabinet containing 60 L of solution; four additional 150 L nutrient supply units provided solution circulation and replenishment in the system. Like the Hurley System (Clement et al. 1974), the Phyto-Nutri-Tron used automated feedback to control nutrient ion composition. However, solution monitoring was performed using an autoanalyzer (phosphate), flow-injection analyzer (phosphate, ammonia, nitrate, nitrite), and inductively-coupled plasma atomic emission spectroscopy (ICP-AES) for metals and other ions.

Not all systems were this complex, however. Another system was used to study rice plants (Raman et al. 1995a) and used significantly smaller solution volumes of 4.2 L and an ion selective electrode to measure nitrate depletion over approximately 20 minute intervals. Here, nitrate usage was quantified through direct ISE depletion measurement rather than through depletion of replenishment solutions. This system also offered temperature conditioning and controlled lighting to offer better climate conditions as well as the ability to conduct experiments over short time intervals on the order of 20 minutes. At perhaps the most basic level, Zavoda et al. (2001) used a recirculating trough system with a total system volume of 4 L to expose either dwarf sunflower or *B. juncea* to various organic and heavy metal contaminants, including Cd. Here, the system featured no instrumentation and plant tissue was analyzed for contaminant uptake at the end of the experiment. In terms of ion uptake, other

systems (Caldwell et al. 1978; Ben-Yaakov and Ben-Asher 1982; Glass et al. 1987; Liao et al. 2000b) were found that varied in complexity between these extremes.

2.4.2. Flow through systems and Cd

Cd uptake through use of flowing culture systems was not common throughout the literature. The Hurley System was used in three reports studying Cd uptake (Jarvis et al. 1976; Jarvis and Jones 1978; Hatch et al. 1988). Jarvis et al. (1976) examined Cd uptake in 23 species through analysis of plant tissue by atomic absorption spectroscopy (AAS) and depletion of Cd from solution in isolated 1 L containers. They used concentrations between 0 and 2.23 μM Cd and found that most of the species tested preferentially accumulated Cd in the roots. They also found evidence for nonspecific adsorption to ryegrass roots and competition effects of Ca, Mn, and Zn for Cd uptake. Furthermore, roots killed by a two minute exposure to boiling water exhibited higher Cd uptake in the presence of a 45-fold excess concentration of Ca than live roots (Jarvis et al. 1976). Another study (Jarvis and Jones 1978) examined the effects of a 15 day exposure to 0.09 μM Cd on 4, 5, 6, or 7 week-old ryegrass plants grown in the Hurley System. They found that root uptake was rapid during the first three days and relatively constant thereafter, regardless of age. Additionally, over 90% of the Cd was root bound, and young plants had higher shoot concentrations than older ones at the end of the 15 day exposure (Jarvis and Jones 1978). Their data also appeared to suggest a Cd induced stimulatory effect on growth, although their report did not address this and the statistical significance of the data was unknown. Hatch et al. (1988) used the Hurley System to examine the effect of varying solution pH from 5.0 to 7.0 on Cd uptake by four species—two species of grasses as well as lettuce and watercress. In all species, total Cd uptake from the 0.18 μM Cd solution was

positively correlated with increasing pH. The two grass species generally accumulated higher root Cd levels than the two dicots. Shoot Cd levels also differed between the species; watercress, in particular, accumulated shoot levels that were 3-fold higher (118.5 mg/kg DW) than the nearest competitor at pH 7 (Hatch et al. 1988). Reduced plant Cd accumulation at lower pH values was attributed to a competitive effect of H^+ ions for absorption sites.

2.4.3. Flow through systems and Cd measurement techniques

Notably, in the previously described studies Cd uptake was measured by harvesting plant tissue and subjecting it to some form of atomic absorption or emission spectroscopy in order to ascertain plant Cd levels. However, Cd uptake in flow through systems can also be calculated through other methods, such as monitoring depletion. For these purposes, depending on the concentration employed, radioisotope monitoring (^{109}Cd), AAS units, or Cd ISEs could be used. Each method has certain advantages and disadvantages. Use of radiotracer techniques was relatively common but seemed restricted to studies of individual transporters or examination of plant Cd uptake in small volume batch-type rather than flow through systems. For instance, Zhao et al. (2002) used depletion of ^{109}Cd in 50 mL cultures to calculate uptake kinetics and apoplastic binding in *T. caerulescens*. Technically, use of radiolabeled Cd would certainly be possible in flowing solution cultures, but the large volume of liquid employed in some systems might make this method prohibitively expensive. Also, radioisotope work is inherently difficult, both logistically and experimentally, particularly for large volumes or amounts. Detection limits, however, are quite good, and are only limited by the sensitivity of the counter employed and the half life of the isotope.

Atomic absorption spectroscopy, including all of its various forms (ICP-AES, etc.) was another commonly employed method. Use of this technique was most prevalent in studies analyzing plant tissue or soil Cd levels after acid digestion. This form of measurement avoids the difficulties associated with radioisotopes and would be suitable for in-line use for flowing solution systems. Indeed, this was the method employed by the Phyto-Nutri-Tron (Lorenzen et al. 1998) as a method for quantifying system ion levels. Jarvis et al. (1976) also used AAS to quantify solution Cd levels, but they used discreet sampling techniques; the spectroscopy unit was not part of the flowing culture system. The main problem with AAS and related technologies appeared to be the relatively high cost(s) associated with even basic units as well as the construction requirements of installation and the technical challenges associated with operating the device, particularly one destined for integration into an automated system. Detection limits with these technologies are very good, with limits ranging down to levels below 1 part per billion (ppb), depending on spectroscopy type. Alternatively, Cd ion selective electrodes could be used for depletion measurement in a flow-through system. ISEs offer the advantages of relatively low cost and ease of implementation and use. However, care must be taken to avoid the presence of interfering ions, such as Cu, and ISEs require frequent calibration in order to minimize the effects of noise and drift. Unfortunately, they are not as sensitive as the other methods, although sensitivity is still relatively good with a concentration detection limit of 0.1 μM for Cd ISEs.

In addition to the type of measurement method employed, other factors were important to consider when using flowing culture depletion measurement systems. In a review, Wild et al. (1987) summarized these aspects. Notably, in flowing culture systems a dynamic, rather than static, state exists. Uptake patterns of K and NH_4 showed diurnal variation (Wild et al. 1987) and system biomass was not constant due

to growth. The method of nutrient supply—either continuous or intermittent—was shown to influence NO_3 uptake and hence nutrient supply method could impact heavy metal uptake as well. Additionally, pretreatment was shown by other investigators to impact NO_3 uptake (Raman et al. 1995b) parameters, and Fe deficiency impacted Cd uptake kinetics in *T. caerulescens* (Lombi et al. 2002). Therefore, a plant's prior nutrient history can have a direct bearing on observed uptake rates, generally through the number and type of transporters expressed on the plasma membrane surface.

2.4.4. Nonspecific uptake

Another important aspect that required consideration in uptake studies was nonspecific apoplastic binding, particularly in the case of divalent heavy metals such as Cd. Several authors have noted a linear component to heavy metal uptake kinetics (Salt et al. 1995b; Hart et al. 1998a; Hart et al. 1998b; Lombi et al. 2001b; Lombi et al. 2002) while linear trends may have been present in the data of others (Homma and Hirata 1984; Salt et al. 1997). This linearity was attributed to nonspecific apoplastic binding by divalent heavy metals (Hart et al. 1998a; Hart et al. 1998b). However, an alternative hypothesis suggested that linear kinetics may represent nonspecific, low affinity symplastic ion uptake or an apoplastic pathway to the xylem (White et al. 2002), mainly on the basis that root maximal velocity (V_{max}) was sometimes insufficient to account for shoot Zn levels in *T. caerulescens*. This view, though, was strongly disputed (Ernst et al. 2002), and it was suggested that White et al. (2002) used unrealistic assumptions in their modeling and that metal competition experiments supported a minor, if any, role for an apoplastic pathway to the xylem. Regardless of the interpretation, in a flowing culture system where depletion measurements were used as a method to quantify uptake, consideration of nonspecific root binding was

important in order to distinguish this type of binding from depletion due to plasma membrane transporters.

Various authors have attempted to quantify apoplastic heavy metal binding using different techniques. One study on Cu uptake in ryegrass (Thornton and Macklon 1989) noted that the primary method of managing this problem was through the use of desorption solutions. However, this was suggested to be arbitrary in that it depended on the type of desorbing cation used, its concentration, the chemical nature of the desorbant's counterion (organic or inorganic) and the amount of time used for desorption (Thornton and Macklon 1989). Short time periods necessitated the use of high concentrations of desorbant while potentially ignoring adsorbed Cu fractions requiring a longer exchange time. Therefore, these authors used liquid nitrogen to kill root tissue and study its adsorptive properties. Interestingly, they showed that dead and live roots exhibited very similar Cu uptake isotherms with a fast phase during the first 17 hours followed by a slower phase lasting through 42.4 h, the duration of their experiment. After 19 hours, differences between live and dead roots were statistically significant at 95% confidence levels (Thornton and Macklon 1989), although considerable scatter existed in the difference data. Of particular interest was the inability of dead roots and homogenized cell wall isolates to achieve Cu saturation over time periods of up to 50 hours and the apparent dominance (in terms of magnitude) of these components when calculating total Cu uptake.

Cataldo et al. (1983) investigated Cd uptake using $^{109}CdCl_2$ in soybean plants and divided absorbed Cd into three distinct fractions: (1) total adsorbed Cd, (2) readily exchangeable Cd that exchanged within 90 min in the presence of a high external Cd concentration, and (3) non-exchangeable Cd. To determine the non-biologic uptake, plants underwent a 30 min pretreatment with the metabolic inhibitors 2,4-dinitrophenol (DNP), sodium pentobarbital, and sodium azide (NaN_3) to eliminate

uptake into the symplasm. They found that nonreversible adsorption was the dominant form of uptake at Cd concentrations above 1 μM. However, biologic uptake may not have been completely abolished by the inhibitors (Cataldo et al. 1983), and lack of another method of comparison for determining adsorptive values made drawing firm conclusions on their adsorptive numbers difficult. For instance, DNP, the most effective inhibitor, reduced uptake rates by 78% at .05 μM Cd but only caused a 50% reduction at 5 μM Cd. However, if apoplastic binding was expected to represent a larger proportion of total uptake at higher Cd concentrations, these results would be in line with such a hypothesis. In contrast, other investigators (Costa and Morel 1993) found that DNP and NaN_3 produced stimulatory effects on Cd absorption in *Lupinus albus*. A study of Cd absorption by rice seedlings (Homma and Hirata 1984) used 60 minute absorption of ^{109}Cd labeled solutions followed by a 15 minute desorption period with unlabeled solution.

Another study (Hart et al. 1998b) examined Cd uptake of live and methanol:chloroform treated roots of two wheat cultivars at two different temperatures (23°C and 2°C) in an attempt to differentiate apoplastic from symplastic uptake. The killed roots displayed different uptake rates depending on temperature and Cd concentration. Their uptake studies used ^{109}Cd and short, 20 min uptake times followed by 15 min desorption in 100 μM Cd. They found rapid desorption occurred during the first 15 min followed by little desorption through 60 min, the duration of their desorption experiments. Assuming that the linear increase in uptake rates as Cd concentration increased was due to apoplastic binding, they calculated the kinetic parameters K_m and V_{max} by first subtracting out the linear component from the uptake rate data and fitting a curve to the resulting values. Cohen et al. (1998) used similar methodology in studying the impact of Fe status on Cd uptake in pea plants; in their case, Cd binding to methanol:chloroform treated roots resulted in binding isotherms

with higher slopes than what was observed in live root cells. These higher slopes were attributed to the methanol:chloroform treatment exposing more cell wall binding sites (Lasat et al. 1996; Cohen et al. 1998). This may have been the case, but in the absence of direct evidence, the assumption that all linear increases in uptake rates were due to apoplastic binding was open to question, and simply subtracting a linear component from the data may have added uncertainty to the calculated kinetic parameters. Since one would expect live roots to exhibit steeper slopes than dead ones, their results were intriguing; the precise ways in which methanol:chloroform treatments altered the cell wall and its associated binding sites required further study.

Another approach to characterizing cell wall binding was evident in the work of Zhao et al. (2002) on Cd uptake in two ecotypes of *T. caerulescens*. Under the assumption that symplastic uptake was eliminated or minimized at cold temperatures, they conducted uptake experiments at 22°C and 2°C. Their results at 2°C showed a rapid uptake phase followed by a nearly-flat plateau phase, although a linear increase was still evident in both ecotypes throughout this plateau phase. Concurrent studies of La^{3+} uptake at both temperatures suggested that total binding by the apoplast was similar at both temperatures (Zhao et al. 2002), but a statistical analysis of the differences was not presented. Since temperature was the only method used to reduce symplastic uptake, a direct comparison of Cd cell wall binding at 2°C and 22°C was not possible.

Therefore, a variety of methods were used to try and estimate the contribution of cell wall binding to apparent metal uptake by roots. The most common experimental approach involved an uptake experiment followed by a "strong" (high concentration) desorption period. The main problem in this approach was that the desorbant may not have removed all of the adsorbed ion, particularly in experiments that used relatively short desorption times. Longer desorption times would help with

this problem, but they could result in intracellular Cd loss through efflux or leakage (Zhao et al. 2002), which would also result in measurement errors. Although no direct evidence of Cd leakage was found in the literature, Zn compartmental analysis (Lasat et al. 1998), if it indeed measured efflux from the symplasm, suggested that leakage may certainly be possible. Chemical amendments, such as DNP, NaN_3, or DCCD may not completely inhibit symplastic uptake and, in the case of DNP, were even found to possibly result in higher uptake than untreated roots. Methanol:chloroform treatments also appeared to potentially result in cell walls that had higher binding capacities than cell walls present in live roots, which made a direct comparison of treated versus live roots difficult. Additionally, desorption removed nearly all of the bound Zn from methanol:chloroform treated roots, but had less of an effect on live roots, which seemed to indicate that different binding processes were involved between the two root types. Some of this difference may have been due to the lack of a plasma membrane in the treated roots; membrane surface charge was suggested to play a role in metal binding and uptake (Reid 2001), although the contribution of this component has not been particularly well studied. Also, a methanol:chloroform treatment may give better access to intracellular proteins and the stele, which could alter overall binding properties. Another approach was to kill roots using liquid N_2 and then study their uptake properties (Thornton and Macklon 1989). This approach afforded the relative certainty that symplastic uptake was abolished, although how liquid N_2 treatment affected cell wall structure and binding was unknown.

Although cell wall binding was usually implicated as the cause of linear isotherms due to the presence of negative charges in the cell wall, cell membranes were also noted to contain fixed charges resulting from ionization of phospholipid head groups (Reid 2001). These charges could provide another driving force for linear uptake or result in altered transport through the plasma membrane (Reid 2001),

although widespread consideration of plasma membrane charge was not found in the literature. In general, nonspecific binding represented a sizeable percentage of total uptake, which tended to increase at higher metal concentrations. For accurately determining Cd uptake due to plasma membrane transporters, then, this type of binding required quantification.

Other important factors in flowing culture were the mechanisms responsible for delivery of ions to the root plasma membrane. Mass transfer to the plasma membrane occurred through two fundamental mechanisms: diffusion and convection. These mechanisms necessitated consideration since they determined the effective concentration experienced at the root plasma membrane. If diffusive forces dominated, mass transfer limitations would have resulted in different ion concentrations between the bulk solution and liquid near the root surface in the unstirred layer, thus introducing error into uptake measurements. Generally, flowing culture systems were suggested to use high enough flow rates such that mass transfer limitations were avoided (Wild et al. 1987). However, at low ion concentrations, these same authors suggested that mass transfer limitations could be important and would result in non-Michaelis-Menten uptake kinetics.

2.4.5. Cd uptake kinetics and molecular biology

In a flowing culture system, Cd first enters a plant at the root surface. If apoplastic paths are ignored, then uptake into a plant occurs at the root plasma membrane surface through transport proteins. Those proteins that were likely involved in plant Cd transport were reviewed earlier. Molecular transporters, in general, were categorized as follows (Sanders and Bethke 2000):

1. Pumps, which typically utilized ATP to power substrate transport across the membrane, underwent conformational changes and catalyzed scalar (enzymatic) reactions; and
2. Carriers which used H or Na ion flows to power transport, underwent conformational changes during the transport cycle, and catalyzed vectorial (defined as having both magnitude and direction) reactions; and
3. Channels, which relied only on the electrochemical driving force to power transport, underwent no conformational change, and catalyzed vectorial reactions.

Consideration of root biochemistry revealed that carriers and channels were the dominant transporter type for cation uptake at the plasma surface membrane because of the favorable electrochemical potential present across this membrane. In other words, the large inwardly directed electrical gradient—on the order of –200 mV (Clemens et al. 2002)—generated by H^+-ATPases as well as the (likely) smaller inwardly directed concentration gradient favors uptake through channels and carriers. The literature did not reveal a clear role for channels in Cd transport, although a recent review suggested that two types of nonselective cation channels (NSCCs) may have a role in divalent heavy metal transport: (1) depolarization activated NSCCs, and (2) voltage-independent NSCCs (Demidchik and Maathuis 2007). However, more research is clearly needed in order to better understand the biological importance of cation channels in Cd uptake. Carriers were noted to display saturation kinetics that could frequently be modeled using the Michaelis-Menten equation and an overall reaction cycle that was modeled as shown in Figure 2.2 (Sanders and Bethke 2000). Thus, the reaction cycle for a typical carrier involves four distinct steps during translocation of a substrate across a membrane. These types of proteins are of particular interest since they appeared to be the primary way through which Cd

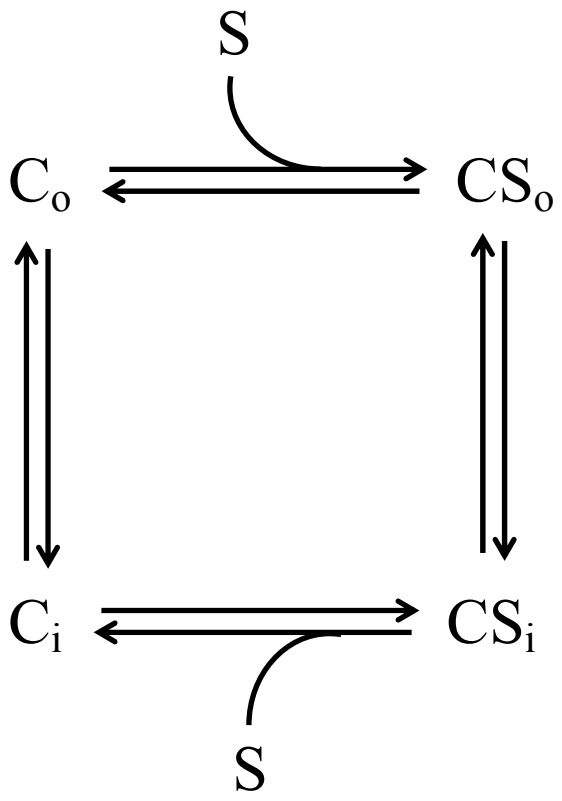

Figure 2.2. Kinetic transport model of a carrier protein translocating substrate across the cellular membrane. C_o, C_i and CS_o, CS_i represent the carrier and carrier-substrate complexes on the outside and inside faces of the membrane, respectively.

entered a plant. Cation channels, such as the Ca channel, represented other potential entry points through the plasma membrane but no direct evidence was found supporting a role for this class of proteins in Cd uptake.

Kinetically, plant Cd uptake was found to be somewhat challenging to model. Several reports on Cd uptake kinetics in plants were found (Cataldo et al. 1983; Homma and Hirata 1984; Mullins and Sommers 1986; Costa and Morel 1993; Salt et al. 1997; Cohen et al. 1998; Hart et al. 1998b; Lombi et al. 2001b; Lombi et al. 2002; Zhao et al. 2002). In general, one of two expressions was used to model Cd uptake kinetics. They were:

$$V = V_{\max}\left[\frac{C}{K_m + C}\right] \qquad \text{[Eq. 2.8]}$$

$$V = V_{\max}\left[\frac{C}{K_m + C}\right] + aC \qquad \text{[Eq. 2.9]}$$

where

V = Cd uptake velocity (moles/h)

V_{max} = maximal Cd uptake velocity (moles/h)

C = Bulk Cd concentration (moles/L)

K_m = kinetic constant (moles/L)

a = slope of linear uptake kinetics (L/h)

Eq. 2.8 is the common Michaelis-Menten expression and Eq. 2.9 adds a linear component to this model, which was done to improve fit to the observed linearity in Cd uptake kinetics. The results of these various Cd kinetic studies are summarized in the Table 2.6.

Based on the studies presented in Table 2.6, a widely divergent range of kinetic parameters were found. The highest and lowest values for K_m were 5.2 μM (Salt et al. 1997) and 0.02 μM (Hart et al. 1998b), respectively. For the parameter V_{max}, the

Table 2.6. Kinetic parameters for cadmium uptake in plants

Plant	K_m (μM)	V_{max} (nmole/g. root-h)	Type of root weight (FW or DW)	*a* [from Eq. 2.9] (L/g. root-h)	Authors
B. napus	5.2	27,700	DW	None	Salt et al. 1997
T. caerulescens	1.21-0.93	14-187.6	FW	3.6-4.7	Lombi et al. 2002
T. caerulescens	0.45	143.2	FW	None	Zhao et al. 2002
T. caerulescens	0.18-0.26	33-160	FW	6-11.2	Lombi et al. 2001
Wheat	0.02-0.04	26-29	FW	None	Hart et al. 1998
Rice	0.18 & 2.27	29.5 & 54.2	FW	None	Homma and Hirata 1984
Soybean	0.076 & 1.2	22.9 & 232	DW	None	Cataldo et al. 1983
Corn	0.030 – 0.1	0.4 (pmole/m^2s)	--	None	Mullins and Sommers 1986
Lupinus albus	.0422	0.116	DW	None	Costa and Morel 1983
Pea	0.6-1.5	34-236	FW	None	Cohen et al. 1998

highest value was 27,7000 nmole/g-root DW-h (Salt et al. 1997) while 0.116 nmole/g-root DW-h (Costa and Morel 1993) represented the low end. Thus, K_m varied by a factor of 260 while V_{max} varied by a factor of 238,793. If the study by Salt et al. 1997 was discarded as an outlier, variation in K_m was 113-fold and V_{max} variation was 1,617. Nevertheless, neither distinct patterns nor precise conclusions were able to be drawn from these numbers, although the hyperaccumulator *T. caerulescens* had some of the higher V_{max} values reported. A large part of the variability may have been due to inter-species differences and, in particular, the different methodologies employed. Therefore, while the Michealis-Menten model was useful in quantitatively describing the saturable nature of Cd uptake kinetics, many variables were clearly capable of influencing parameter estimates. These included prior nutritional status, growth conditions, range of tested Cd concentrations, desorption period and type of desorbant, overall cell wall binding and even the methods used to estimate the parameters. Because of this, making comparisons across species and studies was difficult; development of universal procedures in this regard would be helpful.

3. A SYSTEM FOR HIGH TEMPORAL RESOLUTION OF CADMIUM UPTAKE IN PLANTS

3.1. Introduction

Cadmium, (Cd) is among a problematic group of heavy metal contaminants in the environment (di Toppi and Gabbrielli 1999; Schutzendubel and Polle 2002). Although naturally present at low levels due to events such as volcanic eruptions and forest fires (Hutton 1983; Williams and Harrison 1986), anthropogenic activities have resulted in significant releases of Cd into the environment that in some cases dwarf natural sources by an order of magnitude (Williams and Harrison 1986). Therefore, land that has been contaminated with Cd requires cleanup. Using plants for environmental cleanup, termed phytoremediation, represents one option that may have cost and environmental benefits over other technologies for Cd cleanup (Salt et al. 1995a; McGrath et al. 2001; Mulligan et al. 2001; Robinson et al. 2003b; Linacre et al. 2005). However, a better understanding of plant interactions and responses to Cd is needed so more accurate models of phytoremediation systems can be developed.

Flowing culture systems may offer some insights into these problems through explorations of whole plant uptake dynamics and the ability to monitor uptake under a variety of conditions. In essence, they avoid the complexity of soil-plant interactions where a number of highly coupled abiotic and biotic processes are at work in the transport and sequestration of metal. These confounding factors – such as soil temperature, pH, water supply to the roots, nutrient desorption from the soil matrix and subsequent mass transfer to the root surface – contribute to this complexity (Wild et al. 1987), and can complicate modeling plant processes. In cases where the process is not fully understood or characterized, this reduction in complexity may be beneficial

in that it focuses the research on measuring whole plant metal uptake and sequestration.

These systems have been capable of quantifying temporal and spatial nutrient uptake kinetics. Both cation and anion uptake rates can be measured through the use of flowing culture systems. For instance, uptake of ammonium, nitrate, silica, copper, lead, chromium, and potassium have all been measured using flowing culture systems (Jarvis et al. 1977; McGrath 1982; Macduff et al. 1987a; Macduff et al. 1987b; Raman et al. 1995b; Blaich and Grundhofer 1997; Liao et al. 2000a). However, system design and measurement capabilities varied significantly among the different systems. In a review (Wild et al. 1987), two fundamentally different types of flowing culture systems were identified and developed: (1) nonrecirculating systems where nutrient solution flowed to waste and (2) recirculating systems where solution was reused. In general, uptake calculations were based on either depletion measurements of the nutrient solution (Clement et al. 1974; Glass et al. 1987; Bloom 1989; Raman et al. 1995a; Lorenzen et al. 1998) or on analysis of plant tissue (Jarvis et al. 1977; Macduff et al. 1987a; Blaich and Grundhofer 1997; Liao et al. 2000a).

Plant Cd uptake has also been monitored using flowing culture systems (Jarvis et al. 1976; Jarvis and Jones 1978; Hatch et al. 1988). Jarvis et al. (1976) examined Cd uptake in 23 species in a large 300 L system with a few additional experiments conducted in smaller 1 L containers. Their investigation used atomic absorption spectroscopy (AAS) to analyze metal content of plant tissue. Cd concentrations were between 0 and 2.23 μM and results indicated that most of the species tested preferentially accumulated Cd in the roots. Another study (Jarvis and Jones 1978) examined the effects of a 15 day exposure to 0.09 μM Cd on 4, 5, 6, or 7 week-old ryegrass plants and found that root uptake was rapid during the first three days and relatively constant thereafter, regardless of age. Hatch et al. (1988) examined the

effect of varying solution pH from 5.0 to 7.0 on Cd uptake by two species of grasses as well as lettuce and watercress. In all cases, total Cd uptake from the 0.18 μM Cd solution was positively correlated with increasing pH.

In these Cd studies, uptake was measured by analysis of Cd content in digested plant tissue using atomic spectroscopy. However, other measurement techniques have been used to quantify ion uptake flowing culture systems as well. Lorenzen et al. (1998) used an inline ICP-AES unit to monitor growth solution ion levels. Ion selective electrodes (ISEs) were used by Raman et al. (1995a) and Clement et al. (1974) for measuring ion consumption within their respective systems. Use of radiotracers for Cd measurement was also evident in one report of a flowing culture system (Bloom 1989) as well as in a Cd depletion technique (Zhao et al. 2002). However, while use of radioisotopes in flowing culture systems appeared technically possible, large system volumes, high cost, containment, and disposal concerns appeared to preclude their usage in most flowing culture systems. Therefore, ISEs and some form of atomic spectroscopy, whether used inline or through measurement of discrete samples, have been used predominately for measuring nutrient solution ion levels in flowing culture systems.

Here, we report on development and initial work in a trough based system that was called the Experimental Cadmium Uptake Detection System (ECUDS) which was designed for high temporal resolution of Cd depletion in solution caused by *Brassica napus*, a plant with phytoremediation potential. This system was built with the goal of reliably measuring Cd uptake through the use of depletion measurements made with a Cd ISE for later development of better models of whole-plant interactions with Cd, an important objective in engineering more rational phytoremediation systems. For this study, our objectives were to (1) build a reliable plant rearing system, (2) build a hydroponics-based measurement system, (3) establish baseline characteristics of the

system and components, and (4) demonstrate the system was capable of monitoring Cd depletion caused by *B. napus*.

3.2. Materials and methods

3.2.1. Plant growth and rearing

B. napus seeds cv. Quantum were kept under refrigerated conditions at 4°C until needed for germination. Seeds came pretreated with a fungicide to prevent fungal growth and were germinated by placing them in a beaker with approximately 500 mL of distilled, deionized water, similar to the method for germinating *B. juncea* (Salt et al. 1997). This beaker was then covered with a 14 by 14 cm black acrylic square that had a 2 cm diameter hole cut in the top, to accommodate an aeration line, and was placed in a 25°C covered water bath for five days.

After five days, seedlings that had reached a length of 3 cm or more were transferred to the plant rearing tank (Figure 3.1). This tank consisted of a covered black rectangular tub (Harvester 2 X 4, CSL, Inc., USA) with top dimensions of 1.2 × 0.61 m (length × width), bottom dimensions of 1 m × 0.4 m (length × width), and a depth of 16 cm. When full, the rearing unit contained 48 L of nutrient solution. The cover was a 5 mm thick black acrylic (Lucite L, Ineos Acrylics, Inc., USA) sheet in which thirty-two 4.5 cm diameter holes were cut to form eight rows. These rows were centered 14.6 cm apart with individual plant holes centered 13.7 cm apart so that each plant had 200 cm^2 of growing area. Each of the two shorter tub sides had three 6.35 mm diameter barbed bulkhead fittings (Nalge Nunc International, USA) placed 37 mm from the bottom and 14.3 cm apart. Cylindrical airstones (Penn-Plax, Inc., USA) were inserted into the interior ends of the fittings and air hoses were connected to the

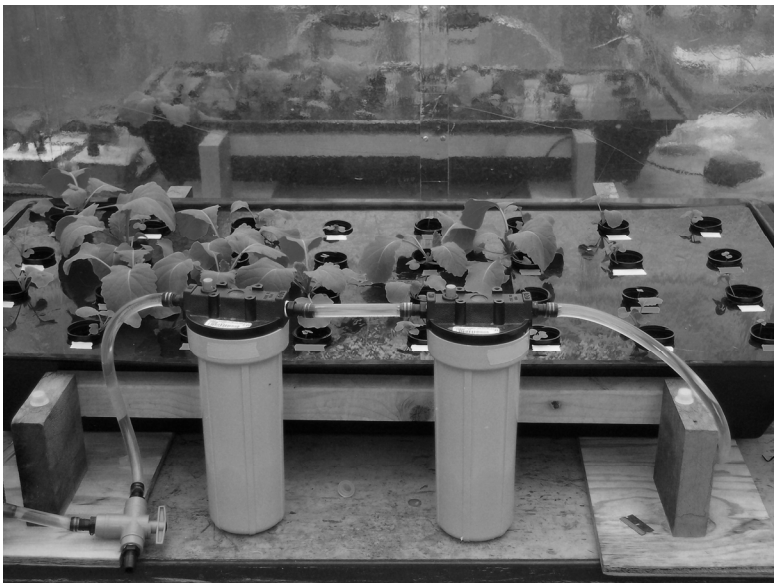

Figure 3.1. Photograph of the plant growth unit used for *B. napus* plants. Nutrient solution was drawn through an exit port (left, not shown) through the filtration units and returned to the growth unit through the re-entry port (right).

exterior side during plant growth in order to aerate the nutrient solution. Two larger 12.7 mm diameter barbed bulkhead fittings (Nalge Nunc International, USA) were inserted into one of the longer sides for the purposes of providing exit and re-entry ports for the nutrient solution. These fittings were spaced 85.1 cm apart, 8.9 cm from each edge, and 4.5 cm above the tub bottom. This tank was kept in a growth room where temperature was maintained between 23–26°C. Lighting was provided by a row of overhead metal halide HID lamps that were set to a 16 h/8 h light/dark cycle. A heat sink (not shown) was constructed of clear acrylic that was mounted on a stand above the rearing tank in order to prevent longwave radiation from killing plant seedlings. This heat sink measured 1.28 m × 0.7 m × 0.15 m (length × width × depth) and was filled to a 4-6 cm depth with deionized water. Light intensity underneath the heat sink averaged 340 μmole m^{-2} s^{-1}.

Nutrient solution was a modified Johnson's solution (Johnson et al. 1957) (Table 3.1). The solution was pumped from the exit port through a filtration system and then returned to the main tank through the re-entry port. Growth solution was filtered through two polypropylene filters (US Filter, Inc., USA) placed in series: one with a 10 μm pore size followed by one with a 1 μm pore size. Filter housings were made of opaque polypropylene (Cole-Parmer, USA) with 12.7 mm (1/2 in.) female NPT ports; appropriate flexible tubing adapters were purchased in order to make the housings compatible with 11.11 mm (7/16 in.) I.D. tubing (VWR, Inc., USA) that directed fluid flow through the filtration system. An impeller driven pump (Model 1046, Eheim, Germany) circulated growth solution through the filters at 606 L h^{-1}, giving media a mean hydraulic residence time of 4.75 min. In order to prevent nutrient depletion, new nutrient solution was added at a rate of 32 L per week. Solution conductivity was monitored twice weekly and kept above 350 μS cm^{-1}. After

Table 3.1. Nutrient solution composition

Chemical	Final concentration
Macronutrients	
KNO_3	1.21 mM (.123 g/L)
$Ca(NO_3)_2 \bullet 4H_2O$	0.786 mM (.1855 g/L)
$NH_4H_2PO_4$	2.14 mM (.2465 g/L)
$MgSO_4 \bullet 7H_2O$	2.41 mM (.5947 g/L)
Micronutrients	
MoO_3	0.107 μM (.0154 mg/L)
KCl	10.7 μM (.7984 mg/L)
H_3BO_3	5.34 μM (.330 mg/L)
$ZnSO_4 \bullet H_2O$	0.429 μM (.07692 mg/L)
$MnSO_4 \bullet H_2O$	0.429 μM (.07243 mg/L)
$CuSO_4 \bullet 5H_2O$	0.107 μM (.02679 mg/L)
Fe-HEDTA	6.0 μM (2.00 mg/L)

23 days of growth in this rearing system, plants were placed into the experimental uptake detection system.

3.2.2. Experimental uptake detection system

This system was also housed in the growth room and was designed to circulate Cd containing nutrient media from a reservoir through a 1.82 m long hydroponics trough (American Hydroponics, USA). A schematic of this system is shown in Figure 3.2. Seventeen 4.5 cm diameter holes were cut into the top of the trough to support 28 day-old *B. napus* plants. Initial experiments indicated that the trough bottom was too broad and did not direct enough nutrient flow over the plant roots, so a 3.8 cm diameter PVC pipe (schedule 40, Genova, USA) was cut longitudinally and placed concave-up along the trough bottom in order to provide better liquid channeling. The trough was held in place, above a table, at an angle of declination of 3-4° to assist fluid flow through the system.

Fluid flow was powered by two Masterflex L/S (Cole-Parmer, USA) peristaltic pumps. The first pump withdrew solution from a reservoir through a solenoid valve and directed it into the trough, which drained into a sensor chamber (Figure 3.3) where Cd measurements were made. This chamber housed the Cd ISE, temperature, and pH sensors. The chamber was placed on a magnetic stir plate and a small magnetic stirring bar provided for continuous mixing of system media while in operation. Thus, the chamber mimicked the behavior of a continuously stirred tank reactor (CSTR). Typical fluid volume in this sensor chamber was 95 mL. The second pump was used to keep fluid level constant at the exit port of this chamber and directed fluid through the return line to a pair of solenoid valves.

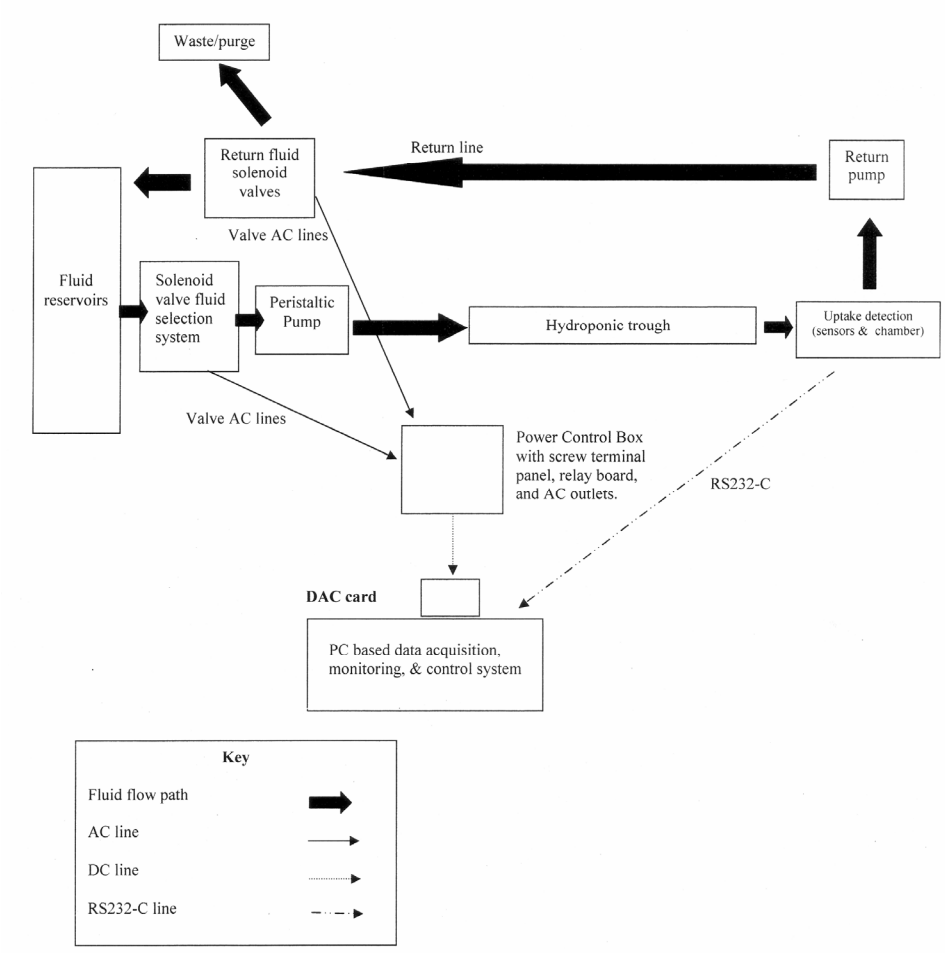

Figure 3.2. Fluid flow and control schematic of the uptake detection system

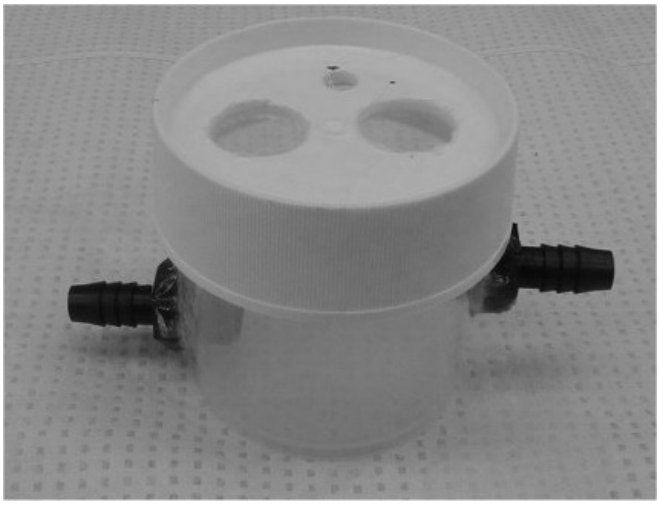

Figure 3.3. Sensor housing chamber with ports for a Cd ISE, pH probe, and temperature sensor. Nutrient solution entered through the lower entry port (left) through gravimetric flow, and was drawn through the exit port (top, right) via a peristaltic pump.

All solenoid valves were controlled by a computer, and two sets of solenoid valves were used to direct fluid flow in the system. The first set of valves was placed between the fluid reservoirs and first peristaltic pump. These valves determined which reservoir the pump would withdraw solution from. A second set of solenoid valves was used on the return line after the second peristaltic pump. These valves determined if return solution was directed back into its original reservoir or if the solution was wasted. The solenoid valve system allowed for rapid change of trough solution if desired.

3.2.3. Data acquisition

A multi-meter (Model 250, Denver Instrument, USA) that provided mV readings with 0.1 mV resolution was used to interface with all system sensors. Analog to digital conversion and noise filtering were handled by the Denver Instrument multi-meter using proprietary hardware. Based on the 0.1 mV resolution capability, the analog-to-digital conversion appeared to be 16-bit. The meter was equipped with a serial port (RS-232) which was used for sending data to a PC running WinWedge Pro 32 (TAL Technologies, Inc., USA) for data acquisition. Data collected in WinWedge Pro 32 was passed to Microsoft Excel 97 using Dynamic Data Exchange (DDE). A program was written in Microsoft Visual Basic for Applications (VBA) that sorted the incoming data and allowed for real-time data display. Solenoid valves were controlled using a data acquisition card (Measurement Computing PCI-DAS 1000) that controlled AC current through the use of optically isolated relays (Opto-22 OAC5). A software program to control the relays was written using a graphical programming interface provided by SoftWIRE (Measurement Computing Corp., USA). All relays,

screw terminal panels, and AC outlets were housed together in a power control box (not shown).

3.2.4. System sensors

Cd was measured using a laboratory grade glass body, double junction, combination ISE (Catalog #A-27502-07, Cole-Parmer, USA) that did not require a separate reference electrode. This sensor has a measurement range between 10^{-1} and 10^{-7} M Cd, making it possible to use this sensor for low level Cd uptake determinations. This ISE is sensitive to both Cu and Fe ions, which are normally present in plant nutrient solutions, and thus all work with this sensor was done in nutrient solutions containing no added Cu or Fe. Such solutions are hereby referred to as minimal nutrient solutions, or MNS. Growth room temperature was kept as constant as was possible in order to minimize the effects of temperature fluctuations on ISE readings. A temperature probe (Denver Instrument catalog # 300733.1) monitored solution temperature. System pH was monitored using a glass bodied combination electrode equipped with automatic temperature compensation (Denver Instrument catalog # 300729.1). All of these sensors were connected to the multi-meter and housed in the sensor chamber described previously.

3.2.5. Cd ISE characteristics

In order to establish a performance baseline for the Cd ISE, numerous experiments with this sensor were first carried out on a laboratory bench top. For these experiments, 200 mL Cd samples were made that used plant nutrient solution to provide the background ionic strength. These samples were kept in 250 mL HDPE

screw-top containers (VWR, Inc., USA) with a magnetic stir bar. These containers were placed inside of 600 mL water jacketed beakers (Schott, Inc., USA) that were partially filled with water. The water jacketed beakers were connected in series to a controlled temperature water bath (Fisher Scientific Isotemp Model 90) that circulated 22°C water through all of the 600 mL beakers. Three 600 mL water jacketed beakers were used in total. Prior to each experiment, the 250 mL containers with Cd were allowed to equilibrate to the set temperature (22°C) for one hour. This setup allowed for very precise control of Cd solution temperature. During experiments, Cd solutions were stirred using a magnetic stir plate. Experiments to determine baseline characteristics focused on sensitivity to low concentrations of Cd as well as response characteristics to changing and unchanging Cd concentrations. Response to unchanging Cd levels examined sensor performance in both short term (1 h or less) and long term (4 h) measurement periods. Several replicates were conducted on a Cd solution in order to obtain error estimates; the ISE was rinsed with DI water and gently blotted dry between replicates. Response to changing Cd concentrations was examined by introducing rapid concentration changes in a stirred Cd solution by rapidly adding nutrient solution that contained no added Cd. To capture the rapidly changing ISE output in these experiments, data download frequency from the multi-meter was set at 1 Hz.

3.2.6. Uptake system characteristics

Performance of the Cd sensor in ECUDS was determined through a series of experiments that used a trough without plants in order to study basic system stability and examine any potential interactions of Cd with system components. These experiments were conducted in the growth room that was described previously. To

establish a baseline, nutrient media containing Cd was circulated through the system. In order to minimize temperature fluctuations, all nutrient solutions had equilibrated in the growth room for at least 12 h prior to use. Once Cd solution was flowing through the system, the ISE was placed into the sensor chamber and readings were taken on the constant concentration solution that circulated through ECUDS for several hours.

3.2.7. Uptake experiments

The general timeline for uptake experiments is shown in Table 3.2. Nutrient solutions were made on day 28 in preparation for the uptake experiment the following day. First, 10 L of nutrient solution was made with the composition as indicated in Table 3.1. This solution was immediately taken to the growth room and set up to circulate through ECUDS. Plants were then removed from the rearing tank and placed into the trough to begin their equilibration period, which was never less than 24 h. Next, 10 L of MNS was made that was buffered with 2 mM MES, pH 6 in order to control ECUDS system pH during experiments. KOH was used to bring this solution to the desired pH. The Cd solution was made by adding an appropriate amount of a 10^{-3} M $Cd(NO_3)_2 \bullet 4H_2O$ stock solution was added to a 2 L acid-rinsed volumetric flask using acid-rinsed volumetric pipettes. MNS was then used to fill this volumetric flask. Cd calibration standards were prepared that included full Cd concentration solution, 50% concentration strength, and 10% concentration strength. All standards were stored in tightly capped, acid-rinsed 250 mL HDPE bottles (VWR, Inc., USA) with a 3.8 cm (1 ½ in.) acid-rinsed magnetic stir bar. Upon completion, all solutions and standards were placed in the plant growth room and allowed to equilibrate for at least 12 h. All glassware was thoroughly washed and acid-rinsed prior to its next use.

Table 3.2. Experimental timeline for *B. napus* plants

Day(s)	Action
1	Germinate seeds
5	Transfer seedlings to growth unit
5-27	Plant growth
28	Transfer plants to uptake detection system with circulating solution; make solutions and standards
29	Run uptake experiment; place plants into drying oven
30	Record plant mass

On day 29, plant uptake experiments were conducted. One hour prior to exposing plants to Cd, system solution was purged and replaced with MNS. This step was used to reduce levels of Cu and Fe to non-problematic levels. During this time, the Cd ISE was calibrated using the standards made on day 28. Exposure time to each stirred standard was typically 15min, which allowed for adequate data collection. Once calibration was complete, the ISE was stored in the final (highest concentration) standard until one hour had elapsed. Then, system solution was immediately changed to a Cd solution, sensors were placed in the sensor chamber, and data collection was initiated.

After completion of an uptake experiment, the ISE was re-calibrated using the same standards in order to capture any instrument drift that occurred. *B. napus* plants were removed from the trough, and the system was thoroughly flushed with 5 L DI water. All system carboys and components were thoroughly washed and rinsed prior to the next experiment.

Roots were separated from above ground portions of the plants and blotted to dryness on paper towels. Roots were then placed in pre-weighed paper envelopes and dried in a 100°C oven for 24 h. Afterwards, envelopes were then weighed again with and without the dried roots; the difference was taken to represent an estimate of root mass. Root weight was used to normalize calculated depletion to a dry weight (DW) basis, and all subsequent mass data should be interpreted on this basis.

3.3. Results

The *B. napus* growth system provided a stable supply of healthy, 28 day-old plants. Typically, growth was staggered so that four differently-aged groups of plants were growing in the system at any one time; in this way, it was possible to have a new

group of 28 day-old plants ready for use on a weekly basis. The two-stage filtration system kept nutrient solution free of particulates. Solution replacement of 32 L per week kept system solution fresh and no nutritional deficiencies were observed as evidenced by the lack of chlorosis, tipburn, iron precipitate formation on plant roots or other visual signs of stress, and therefore maintaining system conductivity above 350 μs cm^{-1} was deemed sufficient for maintaining *B. napus* growth. Typical root DW for individual plants was usually between 0.25-0.35 g. The heat sink absorbed significant amounts of infrared radiation from the growth lamps, and dramatically reduced seedling mortality after placement above the growth system.

3.3.1. Performance of Cd ISE

Sensor output (mV) from some typical short term (45 min), constant Cd concentration laboratory bench top experiments is shown in Figure 3.4(a-b). ISE voltages were generally stable and exhibited good precision, although a sensor equilibration period that was concentration dependent (quicker equilibration at higher Cd concentrations) was present in all experiments. Thus, with the exception of the first 5-10 min, voltage measurements were constant. Mean voltage and coefficient of variation (COV) are shown in Table 3.3 for an experiment that tested three low-level Cd concentrations. COV was greatest at the lowest Cd concentration tested, but decreased at both 0.5 μM and 1 μM.

The Nernst equation, derived from thermodynamics and electrochemical theory, relates electrical response to concentration of an ion (Eq. 3.1):

$$E = E^0 - \frac{RT}{nF}\ln Q \qquad \text{[Eq. 3.1]}$$

where:

E^0 = electrochemical potential under standard conditions, (V)

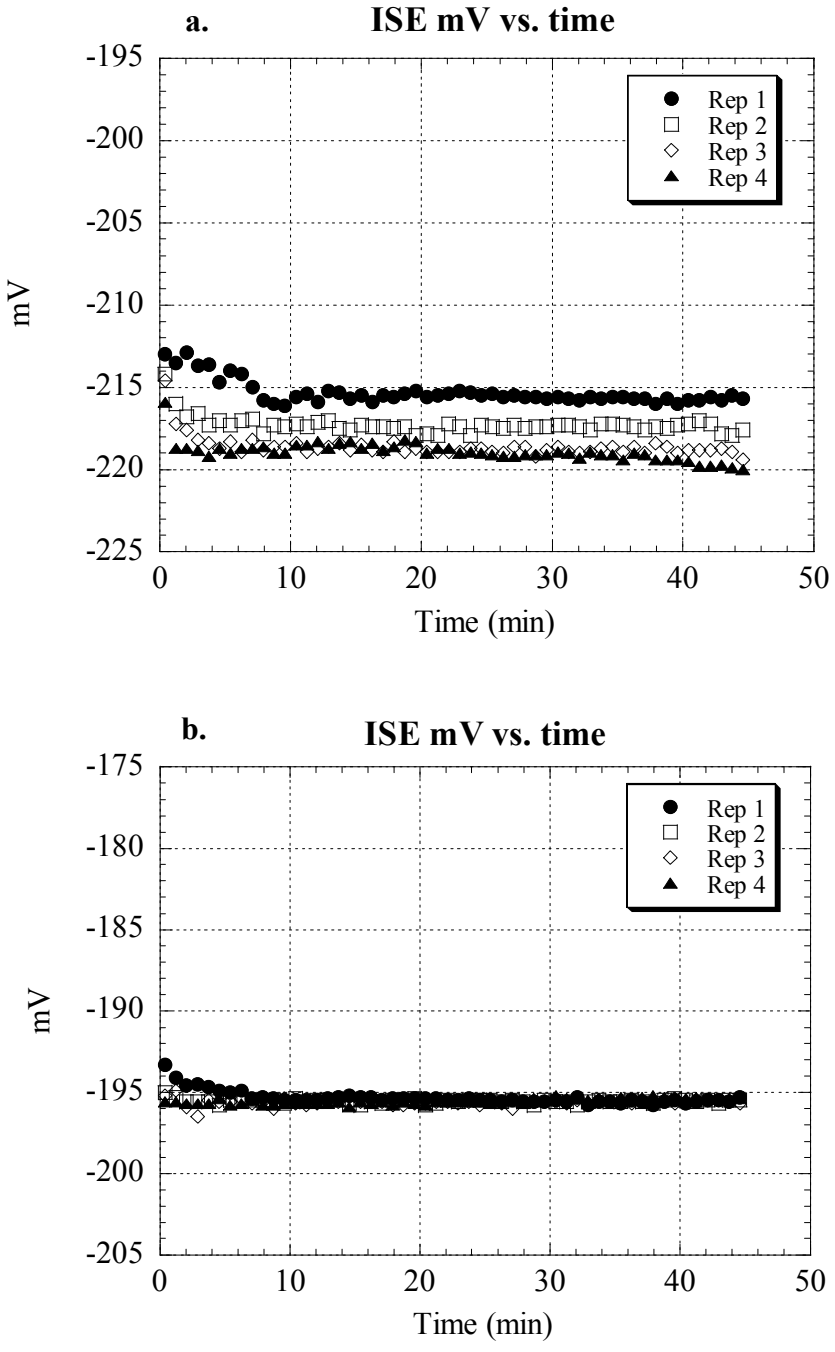

Figure 3.4. Cd ISE response at (a) the limit of detection (0.1 μM Cd) for 45 min, and (b) at 1 μM Cd for 45 min.

Table 3.3. Representative ISE data values

Cd concentration (μM)	*Mean mV value*	*COV*
0.1	-217.6	0.0057
0.5	-204.7	0.0024
1.0	-195.5	0.00059

R = universal gas constant, (J K^{-1} mol^{-1})

T = temperature (K);

n = ionic charge

F = Faraday's constant, (coulomb $mole^{-1}$)

$Q = \frac{1}{[Cd^{2+}]}$ = (L $mole^{-1}$)

At the standard reference temperature of 25°C with a divalent ion such as cadmium, equation 1 predicts a slope of approximately 29.8 mV per ten-fold change in concentration. However, a departure from log-linearity was observed for the sensor at low-level Cd concentrations, which was expected based on literature supplied by the manufacturer. The calculated slope of 22.1 mV obtained from the calibration experiment above deviated from the predicted value by approximately 26%, but was still within device specifications. However, as the sensor aged, sensitivity was reduced which resulted in a decreased slope to around 13 mV/decade (or worse) between 0.1 and 1.0 μM Cd (data not shown). These problems were addressed through the use of three point calibration curves as at the low Cd concentrations used in ECUDS to better capture the changes in sensitivity and the nonlinear behavior of the ISE. A second-order polynomial was then fit to the data using KaleidaGraph 3.6 (Synergy Software, Reading PA). A representative calibration equation is shown in Eq. 3.2:

$$mV = -214.22 + \left(3.47 \times 10^{+7}\right)\left[Cd^{2+}\right] - \left(1.39 \times 10^{+13}\right)\left[Cd^{2+}\right]^2 \qquad \text{[Eq. 3.2]}$$

where:

$[Cd^{2+}]$ = Cd concentration (moles L^{-1})

A numerical solution was computed using Microsoft Excel or MATLAB. In general, a 1 mV change represented approximately a 0.045 μM change in Cd concentration between 0.1 and 1 μM. By using three-point calibration curves and

performing regular maintenance of the electrode, it was possible to keep track of sensitivity and thus know when polishing or replacement was required.

Experiments that investigated longer term ISE stability (4 h) in stirred, controlled temperature solutions showed a stable response over the 4 h period with a drift of about 1 mV (Figure 3.5). Data from four replicates of a 4 h constant concentration experiment are shown in Table 3.4. The first column represents the time at which data was analyzed from the ISE. The mV values reported across the replicate columns at each time point represent the average sensor signal during a 0.5 min window before and after the time point—i.e. the 10 min value is the mean sensor reading between 9.5 and 10.5 min. Next, the change in sensor output over time is reported by calculating the slope of the line drawn between data at two successive time points, and normalizing this data to an hourly value. Although the normalized drift between 10 and 20 min was large, by the time the ISE had been exposed to a given concentration for 10 min, a stable reading had been reached as indicted by the similar drift values between 10-240 min and 20-240 min. Across the four replicates, confidence intervals ($\alpha = .05$) were approximately 2.4 mV at 20 min, which represented a measurement certainty of approximately 0.1 μM Cd, or approximately 10% at concentrations around 1.0 μM Cd. At 240 min., confidence intervals ($\alpha = .05$) had tightened to 1.7 mV, or a measurement certainty of approximately 0.08 μM Cd.

Experiments were also done to investigate ISE behavior in rapidly changing Cd concentrations. Results from a typical dynamic response experiment are shown in Figure 3.6. Concentration drops of 33, 25, 33, and 44% were sequentially introduced by adding nutrient solution with no added Cd into a stirred, controlled temperature Cd solution, which resulted in the observed voltage drops in Figure 3.6. After the initial equilibration period, the sensor was found to respond rapidly to changes in Cd concentration, typically re-stabilizing after a step change in Cd concentration in about

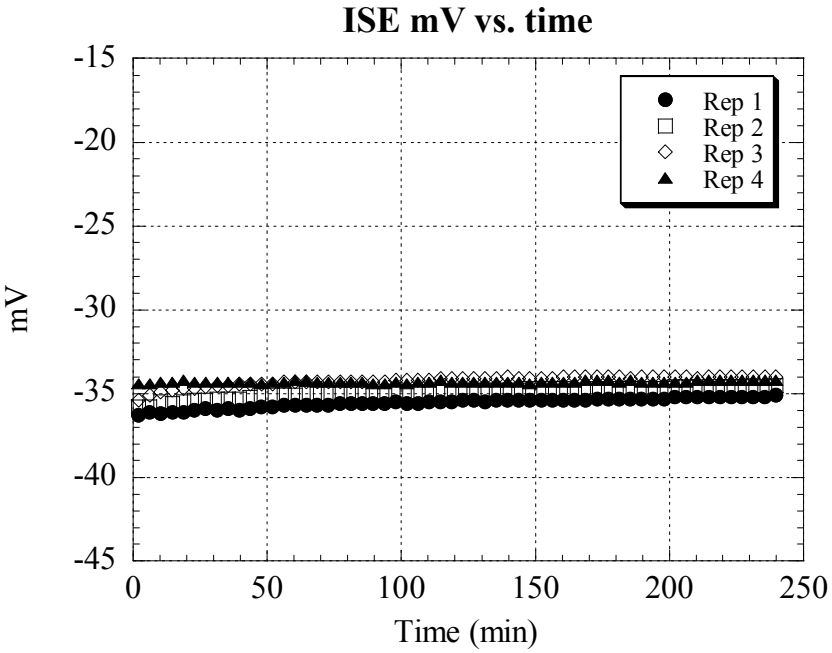

Figure 3.5: Cd ISE stability over a 4 h time period in a stirred, 25°C controlled temperature 0.5 μM Cd solution using MNS for background ionic strength.

Table 3.4. Data from a 4 hour ISE experiment[1]

	ISE mV (Rep 1)	ISE mV (Rep 2)	ISE mV (Rep 3)	ISE mV (Rep 4)
t_1 = 10 min	-36.1	-35.6	-34.9	-34.2
t_2 = 20 min	-36	-35.4	-34.7	-34.3
t_3 = 240 min	-35.1	-34.6	-33.9	-34.2
$\frac{mV_2 - mV_1}{[(t_2 - t_1)/60]}$	0.605	1.21	1.21	-0.605
$\frac{mV_3 - mV_1}{[(t_3 - t_1)/60]}$	0.261	0.261	0.261	0
$\frac{mV_3 - mV_2}{[(t_3 - t_2)/60]}$	0.245	0.218	0.218	0.027

[1] In a stirred, constant temperature nutrient solution with 0.5 μM Cd

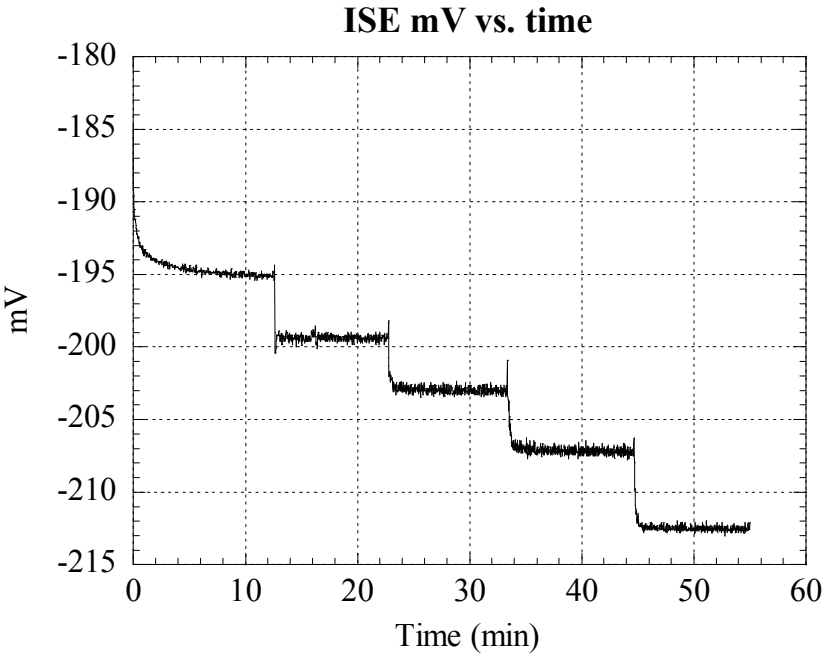

Figure 3.6: Typical dynamic response curve of the Cd ISE in a stirred, constant temperature 25°C solution with MNS providing the background ionic strength.

20 s or less. Also, the introduction of a concentration change was not completely instantaneous; the process of dilution and subsequent mixing took an average of about 5 s, which contributed to the overall electrode stabilization time. Given that concentration drops and fluctuations within the uptake detection system were expected to be on a time scale significantly longer than the apparent minimal response time needed by the ISE (~20s), dynamic response characteristics showed that the ISE would be capable of reacting in time to concentration changes within the system. Due to the lack of sufficiently sensitive equipment, no direct attempt was made to calculate the time constant τ of this electrode, although it appeared to range from less than a second to somewhere on the order of 5 s at low Cd concentrations.

3.3.2. Experimental uptake system performance

To evaluate the baseline stability of ECUDS, experiments were performed by circulating a 1 μM Cd nutrient solution in a trough that did not contain plants. Sensor drift over a time period of 4 h was typically on the order of 1 mV, which translated into an apparent Cd concentration change of approximately 0.045 μM Cd (Figure 3.7). Furthermore, this drift could be managed by regular calibration of the ISE throughout the course of the experiment. Generally, sensor characteristics in ECUDS were unchanged from those observed in the bench top experiments. However, more noise was evident in the trough system based on a comparison of data from 4 h constant concentration experiments done in ECUDS versus those done on the laboratory bench top. By taking sensor data in +/– 30 s windows around 4 time points (10, 20, 100, and 240 min) during the constant-concentration experiments, it was found that the standard deviation of trough data was always higher than that of data from laboratory experiments (Table 3.5). Furthermore, the modified Levene test rejected the null

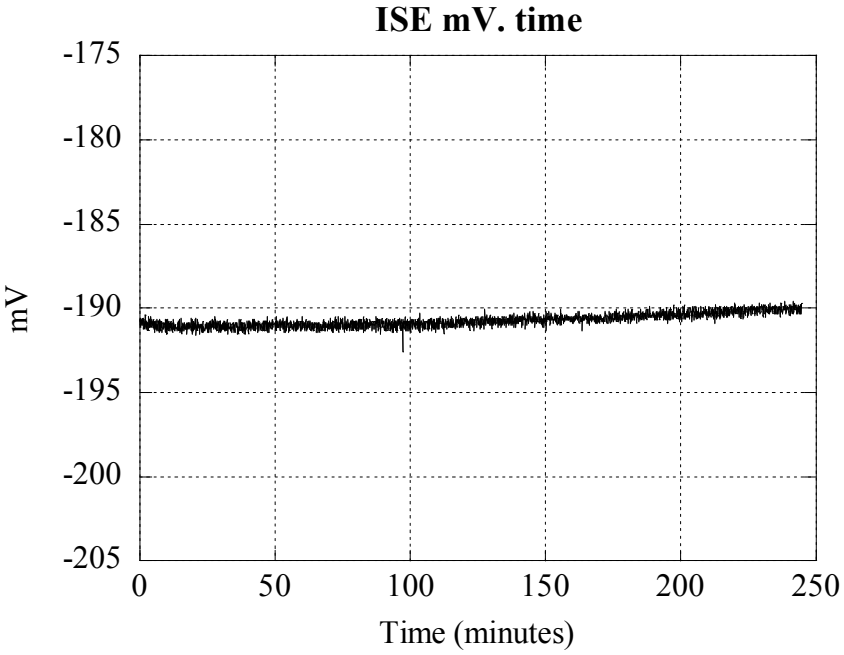

Figure 3.7: Static performance of the experimental uptake system with a 1 μM Cd solution with background MNS over a period of 4 h.

Table 3.5. Standard deviations from trough and laboratory experiments

	Trough		Laboratory	
	Rep 1 (mV)	Rep 2 (mV)	Rep 1 (mV)	Rep 2 (mV)
10 min	0.24	0.27	0.07	0.08
20 min	0.27	0.23	0.10	0
100 min	0.20	0.23	0.08	0.08
240 min	0.26	0.18	0.04	0.03

hypothesis of constant variance between the laboratory and trough data (p = .036). Thus, data from the ISE had more error associated with it when in ECUDS. Some of this additional variability may have also been due to the larger temperature variations observed in ECUDS (data not shown), which was mainly caused by climate control limitations of the growth room. Evaporative losses, while very small, may have also exerted some influence on the observed temperature variations. However, given the stable signal and low amount of sensor drift, ECUDS demonstrated the basic static characteristics needed to detect depletion on the order of several mV or more, provided experiments were conducted over a suitable time scale.

Several experiments were then done using *B. napus* in the trough system. These experiments were done to optimize system parameters that would provide the best system configuration to obtain good uptake measurement results. In the end, a combination of moderate flow rates, sufficient root biomass (6-8 plants; > 1.0 g. total root mass dry weight), and reduced system solution volume (1.7 L) were found to provide feasible conditions for measuring Cd depletion over an approximately 2 h time period. Using these parameters, depletion experiments were conducted in order to find out what kind of uptake rates would be observed by exposing *B. napus* to a 1 μM Cd solution. Results from representative depletion experiments are shown in Figure 3.8(a-b). In these experiments, depletion below the detection limit of the ISE very rapidly occurred over a 2 h time period when the initial Cd exposure concentration was 1 μM. Results presented in Figure 3.8b are from an experiment that used a 2 h pretreatment with 1 μM Cd prior to changing system solutions and taking depletion measurements. Again, very rapid Cd depletion was observed in the system. Additionally, system pH was relatively well controlled with 2 mM MES buffer as ΔpH was approximately 0.12 pH units over the course of the experiment (data not shown). The calibration data from the beginning and end of these experiments indicated the

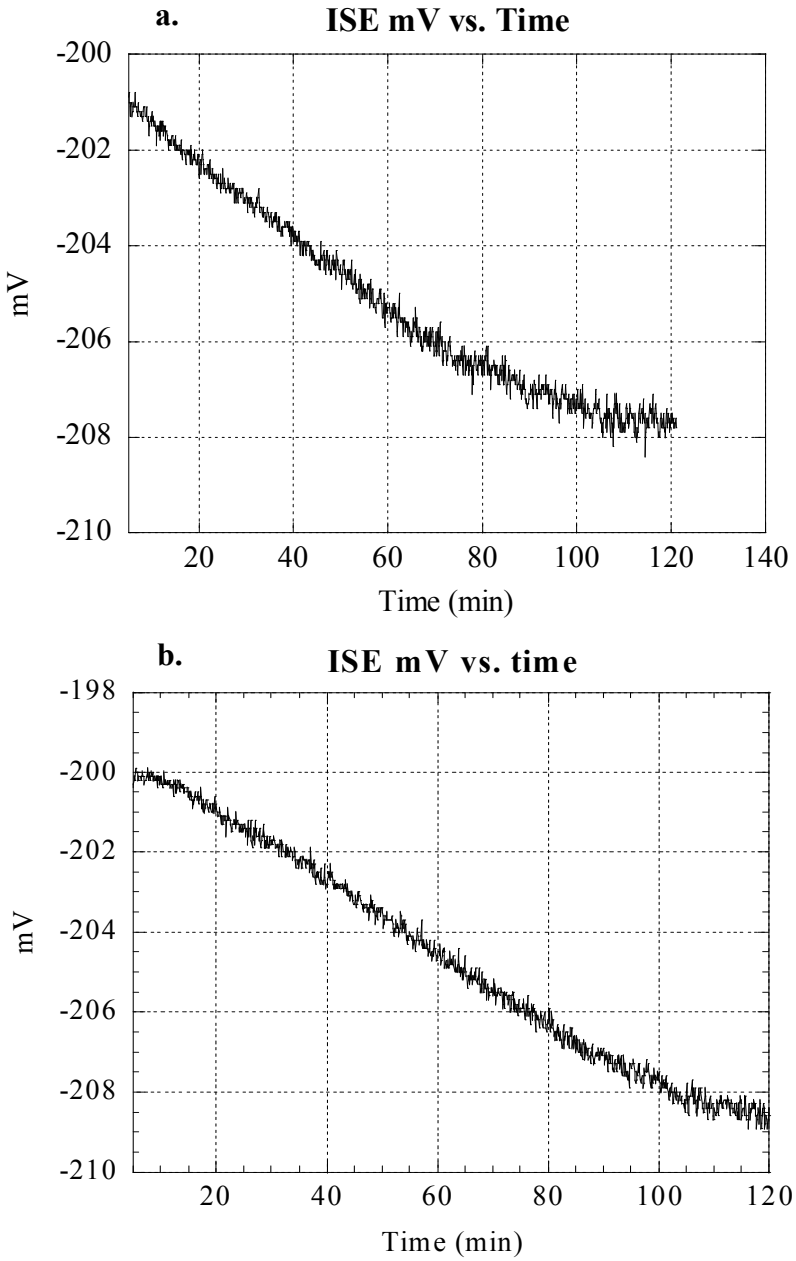

Figure 3.8. Results from some representative depletion measurement experiments using *B. napus*. ISE data from experiments with (a) no Cd pretreatment and (b) a 2 h 1 μM Cd pretreatment. In both cases, the initial Cd concentration of the uptake solution was 1 μM.

cadmium ISE underwent little drift during the uptake period. These experiments demonstrated that ECUDS was capable of detecting cadmium depletion caused by root processes over moderately short (less than 4 h) time periods.

Data shown in Figure 3.8(a-b) were further analyzed to determine a Cd uptake rate. In each case, ISE data from the first 5 min were excluded due to the usual equilibration period of the sensor. Furthermore, data towards the end of each experiment between 100-120 min were also excluded because of curvature that was an instrument artifact as the ISE neared its limit of detection. Therefore, data used for the analysis were between 5 and 77min from Figure 3.8a and between 5 and 95 min from Figure 3.8b. Simple linear regression resulted in a good fit in both cases ($R^2 > 0.99$) and diagnostic residual plots were satisfactory. The resulting equation generated from the linear regression was used to calculate mV values at the beginning and end of each time period covered by a given regression. These values were then converted to Cd concentrations by using the most appropriate calibration curve; therefore, data near the beginning of the experiment used the first calibration curve while data from the end of the experiment used the second calibration curve. In this way, the effects of sensor drift were minimized since the most locally appropriate calibration curve was used. However, the ISE was not able to fully capture the depletion profile over the course of an experiment because of the 5 min equilibration period. Therefore, since the initial Cd concentration was known, it was possible to linearly interpolate between t = 0 and t = 5 min and recreate the depletion profile over time. The Cd concentration profile over time shown in Figure 3.9 was calculated from the ISE data in Figure 3.8b. Two distinct rates of Cd depletion were evident: one between 0-5 min and another between 5-100 min. From Figure 3.9, these rates were calculated to be 1.68×10^{-9} mole Cd s^{-1} and 2.08×10^{-10} mole Cd s^{-1} during these two time periods, respectively. By using the Cd concentration data after 5 min, an overall root uptake rate was calculated over the

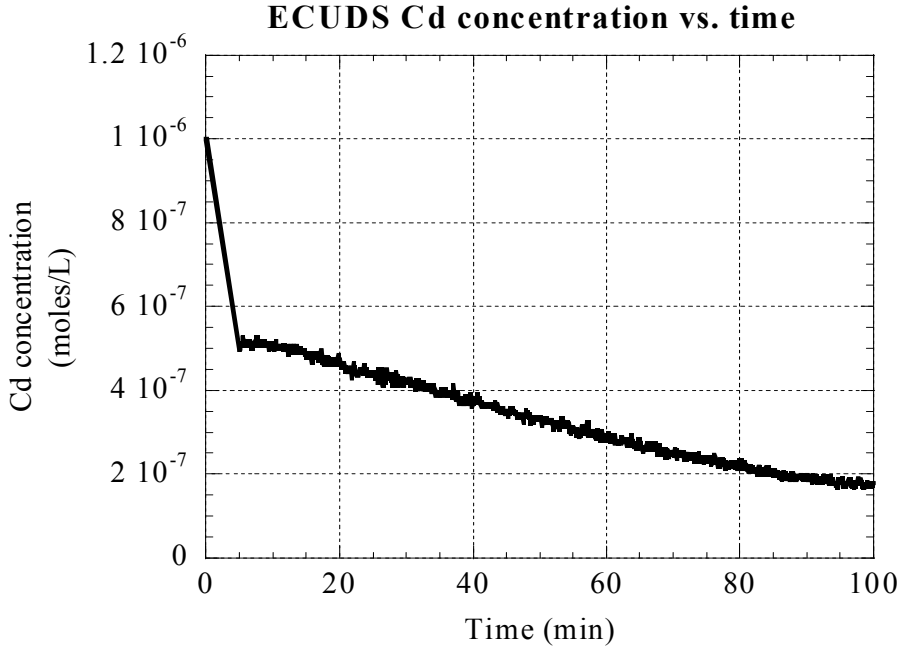

Figure 3.9. Time-course of Cd concentration in ECUDS during the pretreatment experiment.

experimental time period. Uptake values of 1.65×10^{-7} and 1.15×10^{-7} mole Cd g^{-1} h^{-1} were calculated from concentration profiles of the 1 μM Cd uptake experiments with and without a 2 h Cd pretreatment, respectively. This apparent uptake rate fell within the ranges observed in the literature, which suggested ECUDS was capable of obtaining realistic numbers when measuring Cd uptake by *B. napus* (Cataldo et al. 1983; Cohen et al. 1998; Lombi et al. 2002). Additionally, basic functionality of this measurement system was demonstrated for use in future investigations.

3.4. Discussion

With the advent of modern instrumentation and control capabilities, flowing culture systems have been widely used to explore questions surrounding root uptake processes and plant nutrient demands. This type of system may also have utility in development of phytoremediation systems as a way of performance benchmarking and development of plants that are more capable of absorbing nonessential nutrients such as cadmium. However, each type of system requires careful design considerations as to how it will achieve its goal, and what its desired capabilities are, which in turn makes flowing culture system design very specific to the intended task. Furthermore, vigorous characterization of important system components is vital when trying to understand how the system may perform as a whole. Additional issues of electrical noise, environmental variables, solution composition, interfering ions, and sensor limitations are also important. For these design aspects, excellent discussions of such issues are available (Clement et al. 1974; Glass et al. 1987; Wild et al. 1987; Bloom 1989).

Here, we characterized a trough-based flowing culture system with real-time data acquisition and display that used an ISE for detection of Cd depletion caused by

B. napus, a plant that may have use in phytoremediation. Fundamental characteristics of the system and its components, including ECUDS baseline characteristics as well as response behaviors of the ISE were studied. The Cd ISE was found to have relatively low drift (~1 mV) over the 4 h time periods that were of interest to this study when exposed to a constant concentration Cd solution. Furthermore, after an initial equilibration period that generally lasted around 2-10 min, depending on Cd concentration, the ISE was found to respond rapidly to changes in Cd concentration without needing to undergo another equilibration period. This latter feature was extremely useful because of the Cd concentration dynamics that were expected in ECUDS as plant absorption caused Cd depletion over time. Furthermore, drift characteristics allowed for confident calculation of true Cd depletion in the system through simple recalibration of the ISE at various time points.

Early experiments with plants in ECUDS resulted in very rapid drops in system Cd concentration. Within a 2 h period, Cd concentration fell below the detection limit of the ISE. Notably, by the time the ISE had equilibrated to the system solution, the Cd concentration had already dropped from initial levels by approximately 50%. As seen in Figure 3.9, Cd depletion did not occur at a constant rate during the experiment. Rather, a very fast rate of depletion occurred within the first several minutes; exact characterization of depletion during this time period was not possible because the ISE had not yet completed its initial equilibration period after placement in ECUDS. In any event, the Cd depletion rate was dramatically lower by the time the ISE had properly equilibrated to the nutrient solution, as evidenced by the more gradual rate of Cd depletion between 5 and 100 min. Thus, strong evidence for biphasic Cd uptake kinetics was observed in ECUDS. This kinetic profile suggests that two distinct, and possibly unrelated, mechanisms were occurring that caused Cd depletion. Based on characteristics of ECUDS, Cd adsorption to system components

did not appear to be responsible for the observed trend. Root-Cd interactions therefore appeared to be the more likely cause. Additionally, Cd, while capable of entering the symplast, has also been found to bind to the root apoplast (Hart et al. 1998b). This binding is nonspecific and is a source of error that has to be minimized in kinetic studies. Alternatively, differential uptake into the root symplast through regulation of tonoplast transporters could also have caused biphasic uptake kinetics, although regulation of molecular transporters on this short a time scale has not previously been reported. We speculate, then, that nonspecific Cd adsorption to the root apoplast was responsible for the rapid depletion at the beginning of these experiments, although further verification is needed and will be the goal of future work. The lower depletion rate observed at later time periods may have been true symplastic uptake or a combination of reduced adsorption and symplastic uptake. However, basic capabilities of this novel ISE-based Cd uptake detection system have been demonstrated for possible future roles in performance testing of plants with phytoremediation potential.

4. CADMIUM BINDING TO ROOTS OF *B. NAPUS*

4.1. Introduction

Metals such as cadmium (Cd) are able to interact with plant roots in two fundamentally different ways. One such way is through uptake into the symplast mediated by membrane bound transporters. The other interaction, which can be relatively significant, involves nonspecific binding to the root apoplast (cell wall). In fact, this nonspecific binding may be responsible for certain trends that have been observed in several heavy metal uptake studies. Numerous authors have noted that heavy metal uptake kinetics exhibit linear response to bulk Cd loading (Salt et al. 1995b; Cohen et al. 1998; Hart et al. 1998a; Hart et al. 1998b; Lombi et al. 2001b; Lombi et al. 2002). Additional linear trends may have been present in the results of others (Homma and Hirata 1984; Salt et al. 1997), although not as explicitly. It was hypothesized that this linearity response was due to nonspecific apoplastic binding of divalent heavy metals (Hart et al. 1998a; Hart et al. 1998b). However, an alternative hypothesis suggested that linear kinetics represented nonspecific, low affinity symplastic ion uptake or an apoplastic pathway to the xylem (White et al. 2002), mainly on the basis that root maximal velocity (V_{max}) was sometimes insufficient to account for shoot zinc levels in *T. caerulescens*. This view, though, was strongly disputed and it was noted that metal competition experiments supported a minor, if any, role for an apoplastic pathway to the xylem (Ernst et al. 2002).

Despite this controversy over the existence of an apoplastic uptake pathway, nonspecific binding represents a potentially significant source of error that must be carefully managed in plant metal uptake studies in order to obtain good data for uptake models. One study on copper (Cu) uptake in ryegrass (Thornton and Macklon 1989)

noted that the primary method of managing this problem was through the use of desorption solutions. However, the authors believed this method was arbitrary in that it depended on the type of desorbing cation used, its concentration, the chemical nature of the desorbant's counterion (organic or inorganic) and the amount of time used for desorption (Thornton and Macklon 1989). Short desorption periods necessitated the use of high concentrations of desorbant while potentially ignoring adsorbed Cu fractions requiring a longer exchange time. Lengthening the desorption period would address this problem, but then leakage from the symplast could become problematic (Zhao et al. 2002) and result in poor estimates of true uptake into root cells.

A number of methods have been used to gain insight into the nature and extent of apoplastic binding in plant metal uptake studies. In one report that studied Cd uptake in soybean plants, metal uptake was divided into three distinct fractions: (1) total absorbed Cd, (2) readily exchangeable Cd, and (3) non-exchangeable Cd (Cataldo et al. 1983). In order to quantify non-biotic uptake, plants underwent a 30 min pretreatment with the metabolic inhibitors 2,4-dinitrophenol (DNP), sodium pentobarbital, and sodium azide (NaN_3) to eliminate uptake into the symplasm. They found that nonreversible adsorption was the dominant form of uptake at Cd concentrations above 1 μM. However, biotic uptake may not have been completely abolished by the inhibitors (Cataldo et al. 1983), and lack of another method for comparison of binding estimates made verifying their adsorptive numbers difficult. Furthermore, using a cocktail of metabolic inhibitors may not necessarily have similar effects in different species as other investigators found that DNP and NaN_3 produced stimulatory effects on Cd absorption in *Lupinus albus* (Costa and Morel 1993).

Other methods to investigate apoplastic binding involved killing the roots in order to eliminate competing ion uptake into the symplast. One study used boiling

water to kill ryegrass roots and examined Cd uptake in the presence of 100 μM Ca; data indicated that Cd absorption continued for at least 240 min in batch reactors containing 1 L of solution (Jarvis et al. 1976). Another method involved using liquid N_2 to abolish symplastic uptake and therefore provided a measure of Cu binding to the cell wall (Thornton and Macklon 1989). This method involved brief immersions of whole root systems in liquid N_2 to disrupt biological function. Thornton et al. (1989) showed that dead and live roots exhibited very similar Cu temporal uptake responses to bulk Cu loading. Essentially, a fast phase occurred during the first 17 h which was followed by a slower phase lasting through 44 h, the duration of their experiment. After 19 h, differences between live and dead roots were statistically significant at 95% confidence levels (Thornton and Macklon 1989), and thus there existed a basis for differentiating the uptake mechanisms. In addition to liquid N_2, methanol:chloroform treatments have also been used. One study (Hart et al. 1998b) examined Cd uptake of live and methanol:chloroform treated roots of wheat at two different temperatures (23°C and 2°C) in an attempt to differentiate apoplastic from symplastic uptake. The killed roots displayed different uptake rates depending on temperature and Cd concentration, although treated roots at both temperatures exhibited a positive linear uptake response to increasing Cd concentrations (Hart et al. 1998b). The same treatment was also used in a study with peas in order to resolve different Cd uptake components (Cohen et al. 1998). In this study, a second treatment was used which consisted of 0.2 mM $LaCl_3$ in order to block Cd uptake through Ca channels; both treatments resulted in linear Cd uptake isotherms.

Another approach to characterizing cell wall binding was evident in the work of Zhao et al. (2002) on Cd and Zn uptake in two ecotypes of *T. caerulescens*. Under the assumption that symplastic uptake was eliminated or minimized at cold temperatures, they conducted uptake experiments at 22°C and 2°C. Their results at

2°C showed a rapid uptake phase followed by a plateau phase characterized by a very slow or nonexistent period of additional uptake depending upon the metal being tested. Differences between uptake at 2°C and 22°C were taken to represent true uptake into the symplast. For comparison, La absorption, which provided an estimate of apoplastic binding since it did not enter cells, suggested that cell wall binding was similar at both temperatures(Zhao et al. 2002), although a statistical analysis of the differences was not presented.

Notably, in much of the literature that involved heavy metal uptake in plants, the focus was on deriving inward fluxes to the root symplast in response to varying concentrations of the metal of interest. Generally, these studies demonstrated saturation uptake kinetics that were modeled using the Michaelis-Menten equation (Lasat et al. 1996; Cohen et al. 1998; Hart et al. 1998b). In this type of study, decisions are made with respect to the absorptive and desorbtive time periods used prior to analysis of tissue for heavy metal content. Seldom, however, are the temporal binding characteristics of the root apoplast directly studied. For those that have, such as in Thornton and Macklon (1989), apoplastic binding was shown to continue over long time periods and potentially result in subsequent errors made in parameter estimates for symplastic uptake models. Here, we report on some experiments based on a small volume, batch reaction method that were designed to examine temporal responses of Cd binding to root apoplastic material of *Brassica napus*. We further present a simple kinetic model that was derived from basic mass action laws for describing the temporal trends observed in the Cd binding data.

4.2. Materials and methods

4.2.1. Experimental Cd uptake detection system

The experiment conducted in ECUDS with dead roots followed the same general experimental procedures that were outlined previously. The primary change was that roots of *B. napus* plants were placed in boiling DI water for 3 min prior to insertion in the trough. Additionally, 15 mL samples were taken from the system solution at various time points, stored in 50 mL disposable plastic tubes (VWR, Inc.), and analyzed using atomic absorption spectroscopy (Instrumentation Laboratory Model 857).

4.2.2. Batch-based system

This procedure involved using less root mass and smaller volumes of nutrient media than trough-based experiments in investigations of root adsorption properties. Two time scales were investigated: periods between 1 and 15 min and periods between 1 and 24 h. The first series of experiments focused on the longer time periods and investigated Cd adsorption in two different background ionic environments: one containing only Cd and one in media without added Cu or Fe (MNS media) that was identical to the media used in previous trough experiments. The second series of experiments focused on the shorter timer periods and Cd adsorption was measured almost exclusively in a MNS ionic background. Each adsorption experiment used roots that were killed by immersing them in an 800 mL beaker (Pyrex, Inc.) filled with boiling DI water for three min. Roots were allowed to dry on paper towels for 15 min in order to reduce variability in mass between replicates. Roots were sectioned and

weighed on an electronic balance (Mettler-Toledo Model AJ100), with approximately 0.5 g of root matter (wet weight basis) used for each replicate. Root matter was then placed into a 50 mL disposable plastic acid rinsed tube (Falcon Blue Max, Becton Dickinson Labware) that had been filled with 40 mL of Cd solution using a 40 mL acid rinsed volumetric pipette (Fisher Scientific). Tubes were then placed on an 18 rpm tube rotator (Scientific Equipment Products, No. 60448) in order to facilitate mixing and reduce mass transfer limitations to the root surface. For each concentration, negative controls consisted of filling four 50 mL tubes with the Cd solution and placing them on the tube rotator for 24 h.

After tubes had been rotated for the appropriate amount of time (between 1 min and 24 h, depending upon experiment), they were removed from the rotator and 10 mL of the nutrient solution was carefully pipetted into a disposable plastic 15 mL tube (VWR International). For adsorption experiments exceeding 2 h, the 50 mL tubes were first centrifuged at 4,000 rpm in a 21°C centrifuge (Fischer Scientific Marathon 21K/BR) for 3 min in order to reduce root interference with pipetting. After placement into the 15 mL tubes, 0.5 mL of ICP grade HNO_3 (EMD Chemicals, OmniTrace purity) was immediately added to all tubes (4.76% final HNO_3 concentration) in order to prevent Cd adsorption to container walls. Remaining solution was filtered using a vacuum pressure pump (Barnant Co., Model 400-1901) attached to a Büchner funnel and flask assembly with 55 mm filter paper (No. 2, Whatman). Separated root material was dried in a 100°C oven (Fisher Scientific, Isotemp 655G) for approximately 72 h to obtain mass on a dry weight basis, which was the basis that was used throughout this paper. Cd levels in the 15 mL tubes were analyzed using an inductively coupled plasma atomic emission spectrometer (Thermo Jarrel Ash ICAP-60) that had been extensively modified (Rutzke 2002). All samples

were syringe filtered through 0.2 μm 16 mm-diameter nylon filters (National Scientific) prior to ICP-AES analysis.

4.2.3. Adsorption calculations

ICP-AES was used to measure Cd in the bulk solution after exposure to dead *B. napus* roots for varying amounts of time. Thus, direct measurements were performed on free or unbound Cd in the batch system. Since the volume and concentration of the initial solution were known, bound Cd amounts were calculated by using the following mass balance:

$$Cd^{2+}_{total} = V\left[Cd^{2+}\right]_{free} + m_r\left[Cd^{2+}\right]_{bound} \quad \text{[Eq. 4.1]}$$

where:

Cd^{2+}_{total} = total Cd in the system (moles);

$\left[Cd^{2+}\right]_{free}$ = concentration of unbound Cd measured by ICP (mole L^{-1})

V = system volume (L)

m_r = root mass in replicate (g.)

$\left[Cd^{2+}\right]_{bound}$ = Cd bound to *B. napus* roots (mole g. $root^{-1}$)

Thus, total moles of bound Cd were given by:

$$m_r\left[Cd^{2+}\right]_{bound} = Cd^{2+}_{total} - V\left[Cd^{2+}\right]_{free} \quad \text{[Eq. 4.2]}$$

Specific adsorption was calculated as:

$$\left[Cd^{2+}\right]_{bound} = \frac{Cd^{2+}_{total} - V\left[Cd^{2+}\right]_{free}}{m_r} \quad \text{[Eq. 4.3]}$$

Extent of binding was calculated from the following equation:

$$\chi = \frac{m_r\left[Cd^{2+}\right]_{bound}}{Cd^{2+}_{total}} = 1 - \frac{V\left[Cd^{2+}\right]_{free}}{Cd^{2+}_{total}} \quad \text{[Eq. 4.4]}$$

Bound Cd fractions were calculated by subtracting moles of remaining Cd from moles of initial Cd. Volume changes introduced by HNO_3 addition were compensated for in concentration calculations. Specific adsorption was calculated on a dry weight basis in order to make consistent comparisons across experiments.

4.2.4. Modeling of Cd binding

In these experiments, Cd adsorption to *B. napus* roots was modeled under the assumption that roots have a finite number of binding sites and that at any given initial Cd concentration, equilibrium was eventually attained between adsorbed and free Cd. A simple model was developed that described Cd binding to roots in this system with heterogeneous (liquid and solid) phases:

$$[Cd] + [Z*] \underset{k_2}{\overset{k_1}{\leftrightarrow}} [CdZ] \qquad \text{[Eq. 4.5]}$$

where:

$[Cd]$ = Cd concentration in solution (mole L^{-1})

$[Z*]$ = concentration of available binding sites (mole g. $root^{-1}$)

$[CdZ]$ = concentration of bound Cd (moles g. $root^{-1}$)

k_1, k_2 = reaction rates (L min^{-1} $mole^{-1}$)

Furthermore, of the binding sites that participated in the reaction, the following mass balance was assumed:

$$[Z]_{tot} = [Z*] + [CdZ] \qquad \text{[Eq. 4.6]}$$

where:

$[Z]_{tot}$ = total participating binding sites (mole g. $root^{-1}$)

Thus, as the binding reaction proceeded, the number of available binding sites decreased until $[Z]_{tot} = [CdZ]$. If the forward reaction is assumed to dominate, the following equation was used to describe the formation of bound Cd:

$$\frac{d[CdZ]}{dt} = k_1[Cd]_0[Z*] \qquad \text{[Eq. 4.7]}$$

where:

$[Cd]_0$ = initial Cd concentration (mole L^{-1})

By making the appropriate substitution from Eq. 4.6 into Eq. 4.7, the following results:

$$\frac{d[CdZ]}{dt} = \beta_2(\beta_1 - [CdZ]) \qquad \text{[Eq. 4.8]}$$

where:

$$\beta_1 = [Z]_{tot}$$

$$\beta_2 = k_1[Cd]_0$$

Rearrangement of Eq. 4.8 yields the following:

$$\frac{d[CdZ]}{(\beta_1 - [CdZ])} = \beta_2 dt \qquad \text{[Eq. 4.9]}$$

The above expression can then be integrated using the substitution method and evaluated at the limits:

$$-\ln(\beta_1 - [CdZ])\Big|_0^{[CdZ]^*} = \beta_2 t\Big|_0^{t^*} \qquad \text{[Eq. 4.10]}$$

Evaluation of Eq. 4.10 yields the following:

$$[CdZ]^* = [Z]_{tot}(1 - \exp[-k_1[Cd]_0 t^*]) \qquad \text{[Eq. 4.11]}$$

Eq. 4.11 represents a particular solution to the definite integral that was used in subsequent modeling work. This equation predicts a saturating binding response that approaches $[Z]_{tot}$ at sufficiently long times. The model was nonlinear in the

parameters and could not be manipulated algebraically into a linear parameter model. Therefore, nonlinear least squares were used to obtain best-fit values for parameter estimates. The fits were done using the curve fitting toolbox in MATLAB 7.5.0 (The MathWorks, Inc). In order to verify convergence on good parameter estimates, multiple starting values were used along with two different search techniques: trust-region and Levenberg-Marquardt. In both cases, no constraints were placed on parameter values.

4.3. Results

4.3.1. Long-term (24 h) Cd adsorption

An initial experiment using dead roots in the trough system resulted in rapid Cd depletion to very low levels (data not shown). Therefore, the batch-based system was developed and results from long-term Cd adsorption studies (between 1-24 h) in a Cd-only ionic background are shown in Figure 4.1. At each of the three concentrations tested, the Cd concentration of the bulk solution dropped rapidly during the first hour. Furthermore, bulk Cd levels stayed low throughout the remainder of the experiment. Extents of binding were over 0.90, 0.90, and 0.86 for the 10 μM, 5 μM, and 1 μM Cd concentrations, respectively. Results for the long-term Cd adsorption studies that were conducted with a MNS ionic background are shown in Figure 4.2, where bulk Cd concentrations vs. time are shown. In all cases, rapid binding to extents greater than 0.80 was achieved within one h at all tested concentrations. Average binding across all time points for both groups of experiments is shown in Table 4.1. Experiments conducted in MNS exhibited lower extents of binding

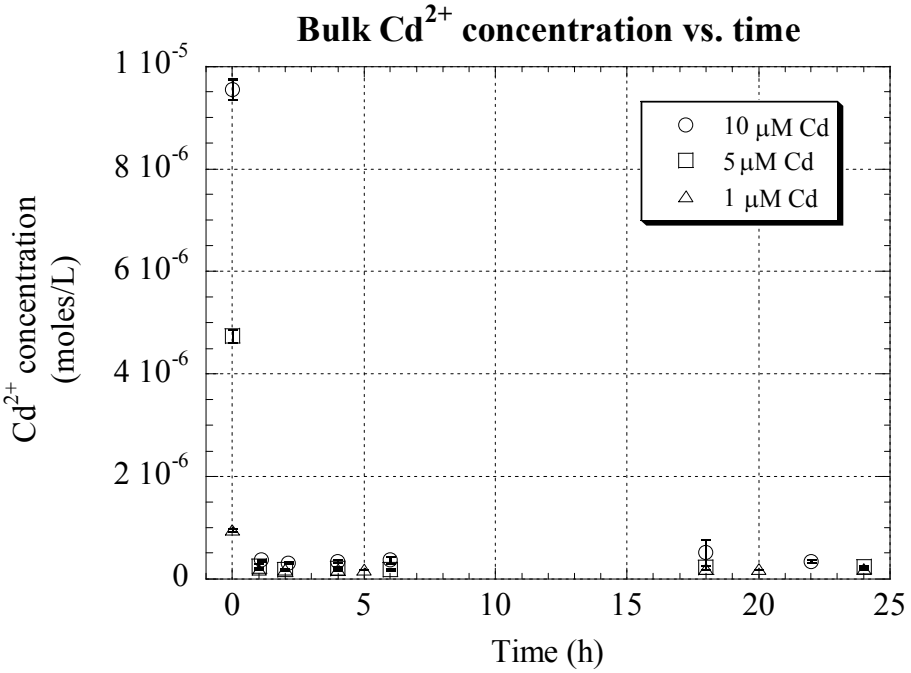

Figure 4.1. Bulk Cd concentration vs. time for the long-term adsorption experiments conducted in a Cd-only ionic background. Error bars represent the standard deviation of the mean.

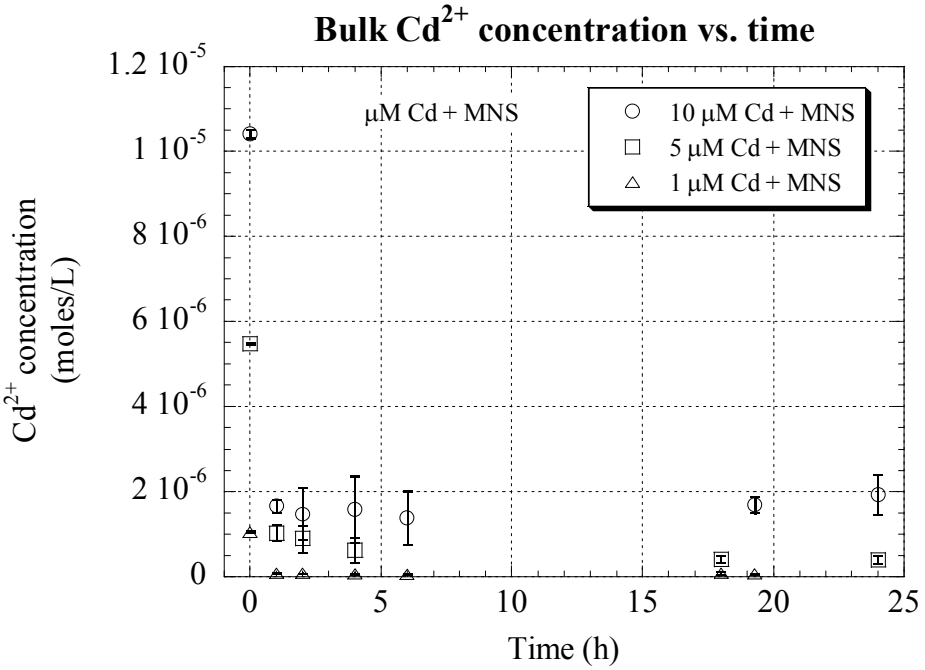

Figure 4.2. Bulk Cd concentration for the long-term adsorption experiments conducted using MNS for the ionic background. Error bars represent the standard deviation of the mean.

Table 4.1. Average extent of Cd binding across all time points for the long-term adsorption experiments.

	Cd background type	
Cd concentration	Cd only	Cd in MNS
1 μM	0.862	0.936
5 μM	0.960	0.878
10 μM	0.971	0.845

compared to Cd-only experiments at 5 μM and 10 μM, while the reverse was true at 1 μM Cd concentrations.

4.3.2. Short-term (15 minute) Cd adsorption experiments

Based on results from long-term experiments, additional Cd adsorption experiments were conducted over a much shorter time period (15 min) in order to better capture adsorption dynamics. Additionally, since the main interest was to understand the type of adsorption that occurred in a multi-element ionic background such as in a hydroponics trough, short term experiments were mainly focused on Cd adsorption to roots in MNS. However, one concentration of a Cd-only solution was tested in the short term adsorption experiments in order to establish a comparison for binding dynamics across the shorter time interval. Again, adsorption experiments used the same MNS composition that was used previously in trough experiments in order to replicate the earlier experimental conditions as accurately as possible. The Cd-only short-term binding results again showed a rapid decrease in bulk Cd concentrations to approximately 0.1 μM as seen in Figure 4.3, similar to the long-term experiments. Here, most of the binding occurred within the first 90 s, the first time point after initial Cd exposure. Results from experiments using MNS for the ionic background are shown in Figure 4.4. In the short-term MNS experiments, average Cd concentrations of the bulk solution at the end of 15 min were 2.0 μM, 0.97 μM, and 0.12 μM for the 10 μM, 5 μM, and 1 μM experiments, respectively. This translated to an extent of binding greater than 0.80 by 15 min at all of the concentrations tested. Notably, bulk Cd concentrations declined more gradually in the MNS experiments and less total Cd was bound when compared to the 10 μM Cd-only experiment, which

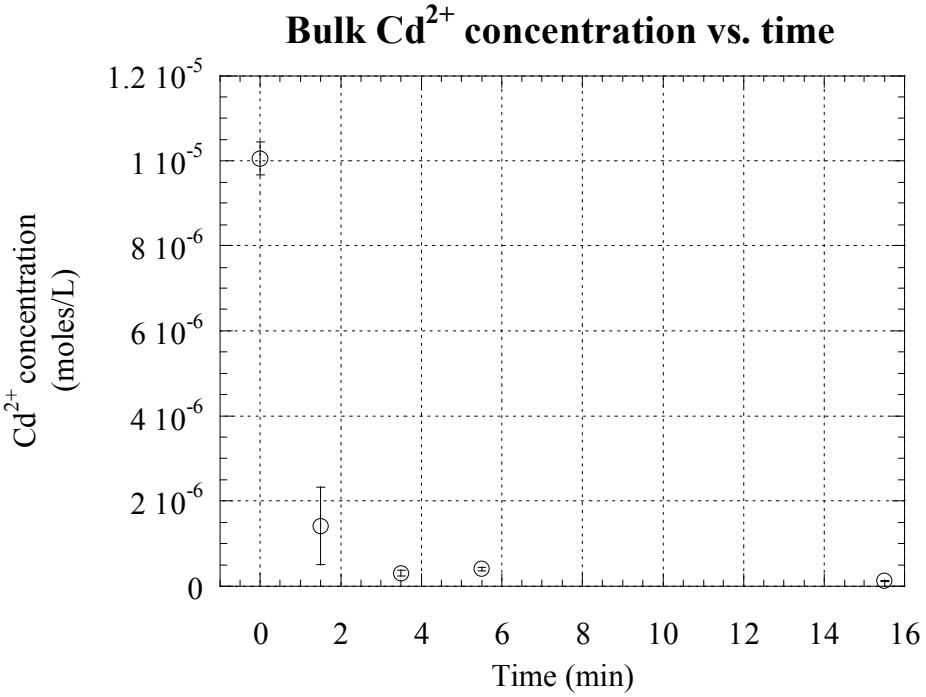

Figure 4.3. Average Cd concentration of the bulk solution vs. time for the short-term experiment using a Cd-only ionic background. Initial Cd concentration was 10 μM. Error bars represent the standard deviation of the mean.

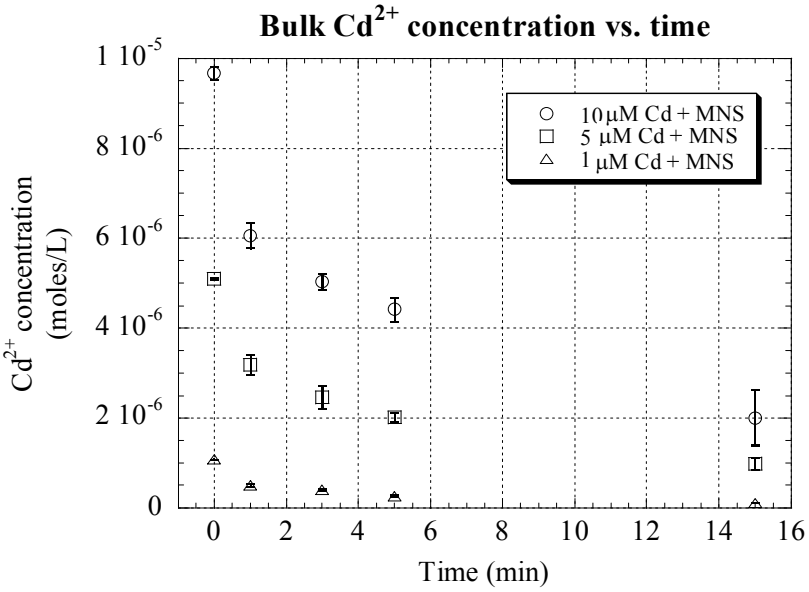

Figure 4.4. Cd concentration of the bulk solution vs. time for the short term adsorption experiments that used MNS for background ionic strength. Error bars represent the standard deviation of the mean.

indicated that the different ionic backgrounds affected the rate and extent of Cd binding to roots of *B. napus*.

4.3.3. Specific bound Cd concentration and effects of root mass

In these experiments, there were two fundamental variables that could influence the amount of Cd depletion. These variables were Cd exposure time and root mass used within the replicate. Exposure time was well controlled, and efforts were made to minimize differences in root mass among the replicates in order to avoid confounding the effects of time and root mass. However, since roots were added to each replicate on a wet weight basis, it was impossible to eliminate variability in root mass among replicates entirely. This variability—basically the water content of the roots—was difficult to control and resulted in a certain spread in the dry weight root mass data. Therefore, it is important to understand how root mass variation and Cd adsorption were used to estimate specific bound Cd in this work. Average root mass and the coefficient of variation (COV) for each time period of the 15 min experiments are shown in Table 4.2. From this table, average root mass of each concentration group was roughly equivalent across the experiments and the median root mass COV was 0.0750. The ratio between the highest and lowest mass values was 1.30 among the MNS experiments, and, as a group, the 10 μM MNS experiment had some of the lowest average mass values as well as the highest COV value of 0.221.

Therefore, in some of the experiments the root mass COV was higher than desired, despite the aforementioned efforts to minimize variation. Nevertheless, this variation did not appear to exert an effect on adsorbed Cd. Table 4.3 shows total adsorbed Cd and specific adsorption, which was the moles of adsorbed Cd divided by the root mass dry weight. Across all experiments, the median adsorbed Cd COV was

Table 4.2. Average root mass data for the 15 min. Cd adsorption experiments.

Initial Cd concentration & background type	Time period (min)	Mean root mass (g.)	COV
1 μM (MNS)	1	0.047	0.035
	3	0.048	0.017
	5	0.046	0.075
	15	0.045	0.049
5 μM (MNS)	1	0.047	0.035
	3	0.048	0.017
	5	0.046	0.075
	15	0.045	0.049
10 μM (MNS)	1	0.037	0.221
	3	0.037	0.184
	5	0.037	0.106
	15	0.048	0.091
10 μM (Cd-only)	1.5	0.034	0.137
	3.5	0.040	0.106
	5.5	0.039	0.136
	15.5	0.038	0.038

Table 4.3. Average moles Cd adsorbed and specific adsorption values for the 15 min adsorption experiments.

Initial Cd concentration & background type	Time period (min)	Mean Cd adsorption (moles)	COV	Specific Cd adsorption (moles/g. root)	COV
1 μM (MNS)	1	2.30×10^{-8}	0.058	4.93×10^{-7}	0.063
	3	2.72×10^{-8}	0.040	5.66×10^{-7}	0.048
	5	3.26×10^{-8}	0.020	7.08×10^{-7}	0.061
	15	3.86×10^{-8}	0.010	8.68×10^{-7}	0.053
5 μM (MNS)	1	7.67×10^{-8}	0.116	2.14×10^{-6}	0.106
	3	1.05×10^{-7}	0.096	2.65×10^{-6}	0.140
	5	1.23×10^{-7}	0.032	2.84×10^{-6}	0.036
	15	1.65×10^{-7}	0.031	3.80×10^{-6}	0.033
10 μM (MNS)	1	1.44×10^{-7}	0.077	4.04×10^{-6}	0.174
	3	1.86×10^{-7}	0.038	5.13×10^{-6}	0.172
	5	2.10×10^{-7}	0.050	5.68×10^{-6}	0.127
	15	3.06×10^{-7}	0.080	6.44×10^{-6}	0.158
10 μM (Cd-only)	1.5	3.46×10^{-7}	0.105	1.03×10^{-5}	0.140
	3.5	3.90×10^{-7}	0.007	9.77×10^{-6}	0.107
	5.5	3.86×10^{-7}	0.004	9.69×10^{-6}	0.115
	15.5	3.97×10^{-7}	0.001	1.06×10^{-5}	0.039

0.039 while the median COV for specific adsorption was 0.107. The 10 μM MNS experiment did not have an unusually high COV for moles of adsorbed Cd even though experiments in this group had high root mass COVs. These results strongly suggest that differences in root mass among replicates and experiments had little influence on how much Cd was adsorbed. However, the effects of root mass variability were evident in the specific adsorbed Cd calculations when COV values are compared: with the exception of one case, specific adsorbed Cd values had higher COVs than total adsorbed Cd. This was particularly true for the 10 μM MNS experiment where specific adsorbed Cd COV values were larger by factors of 2.0 to 4.5. Thus, using the individual root mass of each replicate to calculate specific adsorbed Cd had the effect of generally adding noise to the data. For this reason, average root mass across all time points within a concentration group were used to generate specific adsorbed Cd values for that group which were then used in subsequent plots and model fitting. By calculating specific adsorbed Cd in this way, the original error structure of the adsorbed Cd data was preserved without needlessly adding extra noise to the data.

4.3.4. Influence of initial Cd concentration

Based on the Cd depletion profile that was observed in the 15 min adsorption experiments, initial Cd loading had an influence on the amount of Cd that was adsorbed by the roots after 15 min. This relationship is shown in Figure 4.5. From this, a linear relationship was apparent, and therefore linear regression was used to fit a line to the data. Regression results indicated a good fit to the data ($R^2 > 0.99$), and thus initial Cd loading had a strong influence on bound Cd at 15 min. Initial Cd concentration also influenced the initial rate of Cd depletion, which is shown in Figure

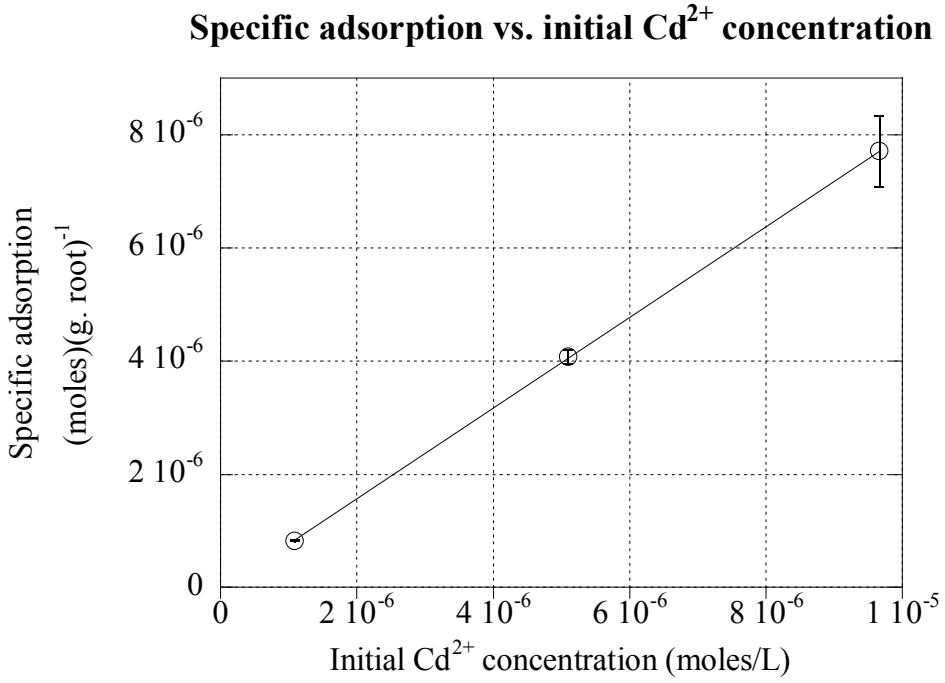

Figure 4.5. Specific adsorbed Cd vs. initial loading concentration for the short term adsorption experiments conducted in MNS. Error bars represent the standard deviation of the mean.

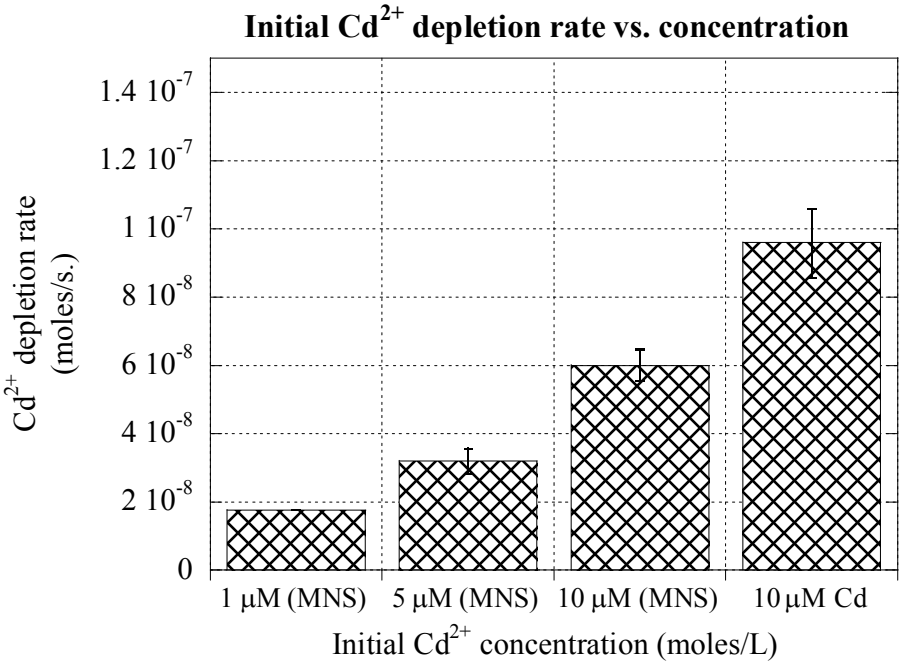

Figure 4.6. Initial Cd depletion rate vs. initial loading concentration. Rate was calculated as the slope between the first two data points in each depletion profile. Error bars represent the standard deviation of the mean.

4.6. Here, rate data was calculated between the starting concentration at t = 0 and the first Cd measurement, which occurred at 1 min for the MNS experiments and 1.5 min for the Cd-only experiment. From this data, higher initial loading concentrations resulted in higher initial depletion rates.

4.3.5. Cd adsorption model results

In order to fit the Cd adsorption model (Eq. 4.8) to the data, bound Cd was first calculated using Eq. 4.3. Specific adsorbed Cd values for a given concentration were calculated using the average root mass of the entire experiment for reasons that were previously explained. Eq. 4.11 was then fitted to the specific bound Cd data over time for each of the Cd concentrations, and results of these fits are shown in Figure 4.7a-b. Because of the nonlinear nature of the curve fitting problem, several different starting parameter set values were tried, and the numeric search technique demonstrated good convergence across these values as it always converged to the same estimated parameters.

Values for parameters and regression fit results are presented in Table 4.4. The model fit the Cd adsorption data well at all tested Cd concentrations, and was able to explain a large amount of variability in each data set as R^2 values ranged from 0.88 to 0.99. The parameter $k_1[Cd]_0$ represented a lumped kinetic parameter that contained both the forward reaction rate k_1 and the initial Cd concentration in solution. This lumped parameter had a relatively constant value for the 10 μM and 5 μM MNS experiments, and higher values for the 1 μM MNS and 10 μM Cd-only experiments. Values of k_1 were also calculated; across the MNS experiments k_1 decreased at higher Cd loading concentrations. Further validation of the model was obtained by a plot of parameter $[Z]_{tot}$ values versus initial Cd concentration for the MNS experiments

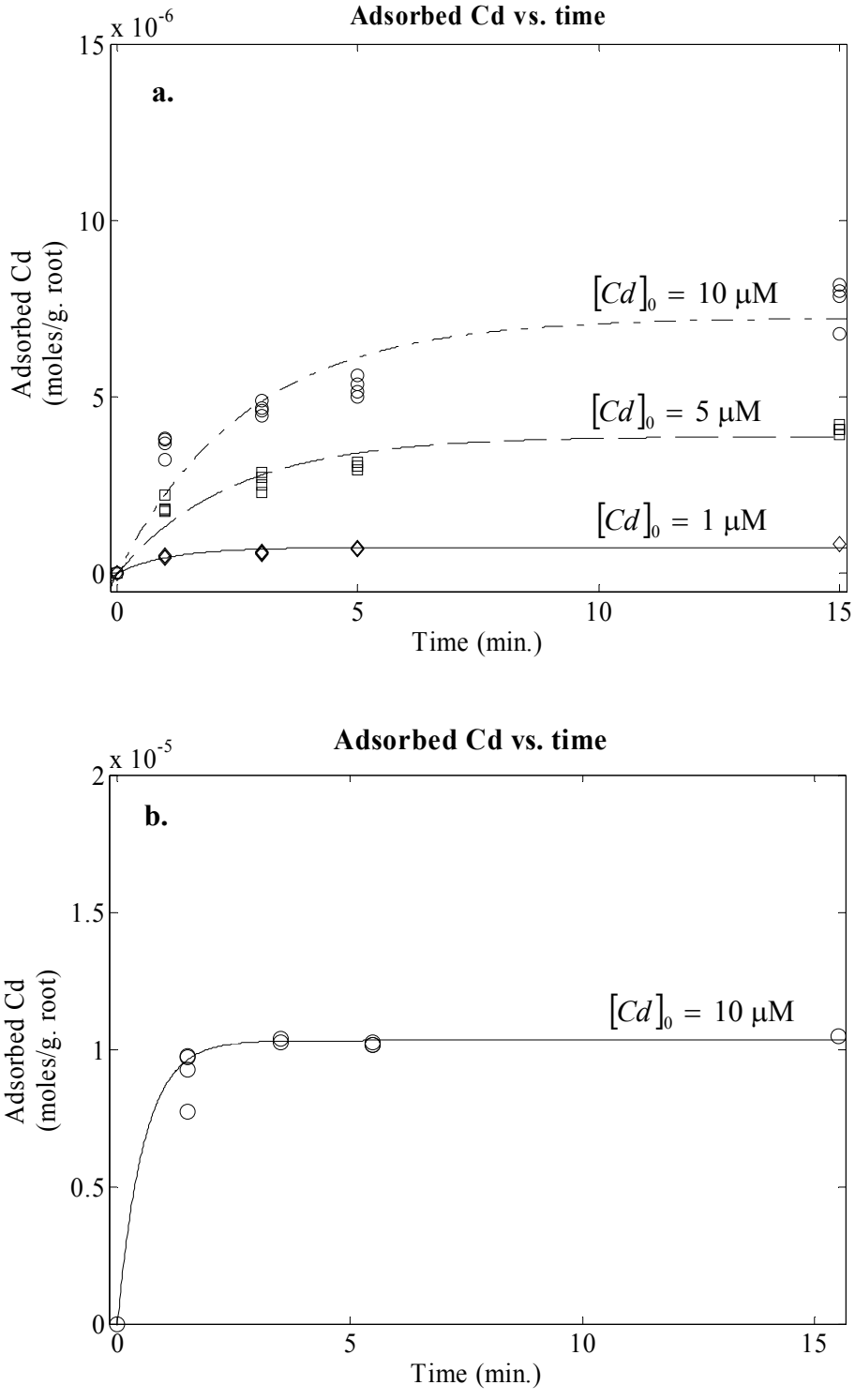

Figure 4.7(a-b). Results of fitting the Cd adsorption model (Eq. 4.11) to experiments that used (a) MNS and (b) Cd-only ionic backgrounds.

Table 4.4. Calculated parameter values and nonlinear regression results for the Cd adsorption model.

Initial Cd loading & ionic background	$[Z]_{tot}$ (moles/g. root)	$k_1[Cd]_0$	k_1 *(L)(min)$^{-1}$(g. root)$^{-1}$*	R^2	SSE
10 μM (MNS)	7.25×10^{-6}	0.372	3.72×10^{4}	0.88	1.50×10^{-11}
5 μM (MNS)	3.88×10^{-6}	0.421	8.43×10^{4}	0.93	2.53×10^{-12}
1 μM (MNS)	7.38×10^{-7}	0.924	9.24×10^{5}	0.92	1.09×10^{-13}
10 μM (Cd-only)	1.03×10^{-5}	1.78	1.78×10^{5}	0.99	8.23×10^{-13}

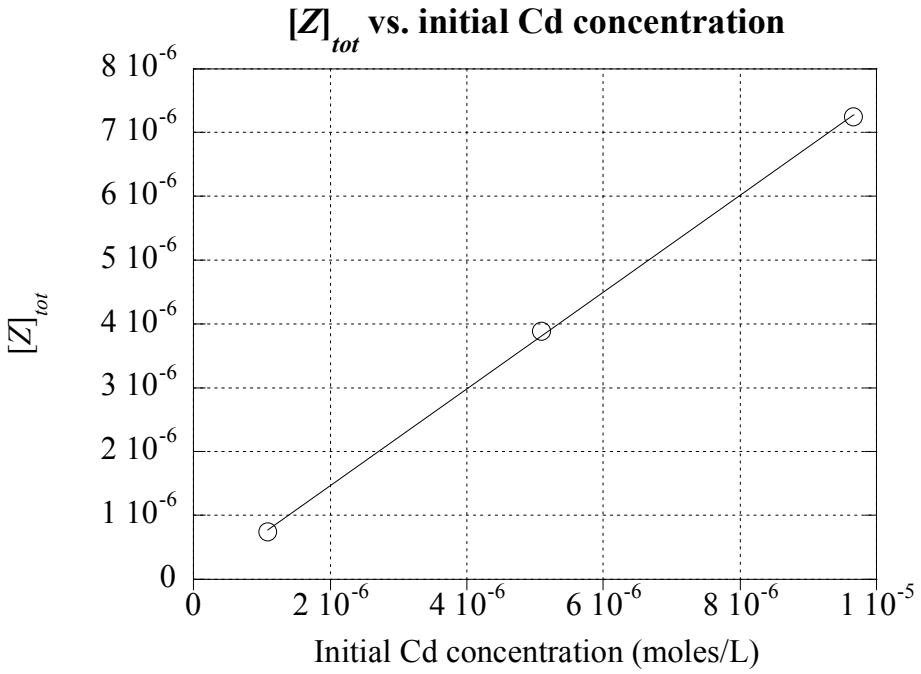

Figure 4.8. Plot of model parameter $[Z]_{tot}$ vs. initial Cd loading concentration.

shown in Figure 4.8. In the case of $[Z]_{tot}$, it was expected that this parameter would be proportional to the initial Cd concentration that was present at $t = 0$. This proportionality is clearly shown in Figure 4.7. To verify this apparent trend, linear regression was used to fit a simple two parameter line to the plotted values. As expected, the relationship was highly linear with $R^2 > 0.99$. This consistency suggested the parameter estimates obtained for the Cd adsorption model through nonlinear least squares were valid. Therefore, Eq. 4.11 was capable of describing Cd binding to heat-killed roots of *B. napus* in both MNS and Cd-only ionic environments.

4.4. Discussion

Nonspecific Cd binding to the root apoplast is an issue that must be addressed when conducting Cd uptake studies. Here, work was presented that explored the nature and dynamics of this binding to heat-killed roots of *B. napus*, and a Cd adsorption model was used to describe the temporal binding patterns of Cd to these roots. Several insights were gained using this batch-based approach to explore this nonspecific adsorption. First, adsorption was rapid and, once bound, Cd remained bound for at least 24 h. Second, short term binding experiments demonstrated that the majority of binding occurred within minutes of Cd exposure, and that differences in background ions influenced initial Cd depletion rates. These differences in initial rates are clearly shown in Figure 4.6, where higher Cd concentrations resulted in higher initial depletion rates. Also, when rates from the 10 μM experiments in different ionic backgrounds were compared, the multi-ion MNS background resulted in a 5.4-fold lower initial depletion rate than the Cd-only background. Finally, Cd adsorption to roots was well described by the temporal binding model presented in Eq. 4.11.

Kinetically, because of approximate linearity in the initial Cd depletion rates for the 15 min MNS experiments, an early attempt was made to model Cd binding as a first-order process. The result was unsatisfactory because the exponential decline in Cd predicted by this model was not observed in experimental data. Attempts at fitting a second order model also led to unsatisfactory results. Therefore, a heterogeneous kinetic model was developed to capture the long term trends in the temporal Cd concentration data. This model, derived from first principles, was capable of describing the process of Cd adsorption to roots over time.

The derived model assumed that the root apoplast had a limited number of Cd binding sites and that the forward reaction dominated while binding eventually attained equilibrium. These assumptions were reasonable: finite root mass implied finite binding sites. Furthermore, the forward reaction did dominate as evidenced by the high extent of Cd binding that was observed. The equilibrium assumption was verified by experimental data since unbound Cd concentrations were detectable and remained relatively unchanged through at least 24 h.

These assumptions are the basic ones made in the Langmuir adsorption model (Langmuir 1916):

$$[CdZ] = \frac{\Gamma_{max} K [Cd]_f}{1 + K [Cd]_f} \qquad \text{[Eq. 4.12]}$$

where:

$[CdZ]$ = amount adsorbed (mole)(g. root)$^{-1}$

Γ_{max} = maximum adsorption (mole)(g. root)$^{-1}$

K = equilibrium constant (L)(mole)$^{-1}$

$[Cd]_f$ = aqueous Cd concentration (mole)(L)$^{-1}$

The Langmuir model (Eq. 4.12) predicts that as $[Cd]_f$ increases, eventually $K[Cd]_f >> 1$ and thus binding reaches Γ_{max}. In this case, too narrow of a Cd

concentration range was tested to verify how well this model would describe Cd binding to roots at high concentrations. However, at the concentrations that were tested, total bound Cd increased linearly with concentration (Figure 4.5). This linear binding response is also predicted by the Langmuir model when $K[Cd]_f \leq 1$, where the following condition holds:

$$[CdZ] \leq \frac{\Gamma_{max}}{2} \qquad \text{[Eq. 4.13]}$$

Therefore, the Cd binding that was observed in this study was consistent with binding in a Langmuir model at concentrations below the level where half saturation occurred. In addition to the linear binding data presented in Figure 4.5, root weight data also suggested these experiments were conducted well below saturation binding concentrations since root mass variability had no apparent effect on Cd binding. Although attempts were made to use the same root mass in each replicate, up to 20% variability in dry weight data was observed in the data. Despite this, adsorbed Cd COVs were low. The apparent lack of an effect for root weight was indicative of a system where binding sites significantly outnumbered Cd ions. This, in turn, provided further support that binding occurred below half-saturation.

Other models have also been developed to describe interactions of cations with the cell wall of plants. In general, these models are based on the fact that cell walls act as ion exchangers and therefore ion interactions can be modeled using classical Donnan theory, or a variation thereof (Dainty and Hope 1961; Demarty et al. 1978; Demarty et al. 1984; Shomer et al. 2003). Another model combined Donnan theory with equilibrium considerations to describe cell wall interactions with Ca, Mg, and K (Sentenac and Grignon 1981). In Donnan-based models, the cell wall is assumed to have a uniform electric field, or, in double-layer theory, a certain distribution of electric charges (Demarty et al. 1978). These models were developed to describe ion

exchange phenomena and the resultant partitioning of ions in the cell wall. Usually, ion exchange experiments for development and verification of these models involved studying the exchange properties of a limited number of ions simultaneously. However, the ionic environment used in the work presented here consisted of multiple ions, which would have resulted in a large number of ion exchanges reactions to model, and thus complicated attempts to extend Donnan ion-exchange models to this system. Alternatively, mass action expressions have also been used to describe ion-exchange properties of cell walls (Bush and McColl 1987). As noted by these authors, using mass action laws avoids two main complications of Donnan conceptualizations: (1) cell wall ionic molalities, which cannot be measured directly by experimental methods, are not needed, and (2) the cell wall is not divided into two volumes (water and Donnan free space) which are difficult to quantify. Instead, the reaction phases are straightforward: aqueous solution and solid ion exchanger. The model presented here, which was also based upon mass action principles, represents an even further simplification from the work of Bush and McColl (1987) in that the nature of the ion exchange reaction was not important. Instead, the focus was on understanding Cd binding to heat killed roots.

Within this framework, the temporal Cd adsorption model (Eq. 4.11) provided a good fit to the experimental data in both MNS and Cd-only ionic environments. Values for the model parameter $[Z]_{tot}$ were in line with expectations (Figure 4.8) as $[Z]_{tot}$ showed a linear response to initial Cd concentration, which agreed well with the linear portion of the Langmuir binding isotherm as described above. Other parameter values, however, were more difficult to interpret. The lumped parameter $k_1[Cd]_0$ represents the value of the exponent in Eq. 4.11 and was reasonably consistent across experiments that used MNS media, although the value was somewhat higher in the 1 μM MNS experiment. The trend in $k_1[Cd]_0$ values for the MNS experiments did not

increase as expected with higher Cd loading rates, which was due to the lack of consistency found in the calculated k_1 values. The lack of consistency, in turn, may have indicated that some of the assumptions made in this modeling approach were not fully met. However, the value for $k_1[Cd]_0$ in the Cd-only solution was noticeably higher, which indicated the exponential term approached zero more rapidly and thus Cd binding reached its maximal value faster. This was roughly in line with expectations since there was less competition for binding sites because no other divalent cations were present in the bulk solution. In the plant nutrient media, other divalent cations such as Zn, Mn, and Ca were present and were likely capable of displacing Cd from some of the binding sites. This competition, in turn, would have led to a reduction in the overall amount of Cd adsorbed over time, which was backed by experimental data and captured kinetically by the Cd binding model.

The concentration range used in these experiments resulted in binding that was consistent with the Langmuir model. The low Cd concentrations used here were chosen deliberately as the main interest was in trying to elucidate the nature of Cd adsorption that had been previously observed in a hydroponics trough. Thus, the focus was understanding temporal trends in Cd adsorption dynamics at concentrations that were at or near environmentally relevant levels and were similar to what was previously used in the hydroponics trough system. By choosing a temporal focus, valuable insight was gained into the binding that might be expected in this larger trough system. Specifically, binding was capable of reaching an equilibrium, which is an important consideration if depletion-based measurement systems are used for measuring uptake of ions that are capable of significant binding to the root apoplast. In these systems, enough Cd must be present so that apoplastic binding does not represent the majority of Cd depletion within the system while at the same time allowing enough detectable Cd to remain in the bulk solution so that other processes,

such as symplastic uptake, can be measured. The batch-based system used here allowed insights into Cd adsorption characteristics while at the same time avoiding the extensive labor and chemical costs associated with running the larger trough system. Therefore, the system demonstrated its utility, although further work remains on verifying saturable binding characteristics at higher Cd concentrations.

5. CADMIUM UPTAKE MEASUREMENTS AND XYLEM SAP THIOL RESPONSES OF *BRASSICA NAPUS*

5.1. Introduction

Phytoremediation involves using plants to clean up a polluted environment. Various forms of this technology have been defined (Salt et al. 1998), but are in relatively early stages of development. One form of this technology, known as phytoextraction, uses plants to remove pollutants such as heavy metals from the soil. Cleanup of both heavy metals and organics may be possible with plant-based remediation systems; however, heavy metals generally cannot undergo further degradation in the soil which in turn makes them particularly amenable to plant-based remediation strategies (Raskin 1996). The ideal plant for heavy metal phytoextraction should have the following properties: accumulation of the target metal to high concentrations, high translocation to above-ground plant biomass, fast growth, high biomass production, and easy harvestability (Karenlampi et al. 2000).

Attempts at using plants for Cd cleanup have been focused on either high biomass crops such as corn or, alternatively, plants called hyperaccumulators that had a natural ability to accumulate high concentrations of metals in their tissues. Problematically, though, high biomass crops frequently displayed undesirable metal accumulation properties while hyperaccumulators tended to grow slowly and have low biomass (Salt et al. 1998; Kayser et al. 2000). In short, our incomplete knowledge of how to most effectively use plants to extract heavy metals from environmental systems has hampered engineering efforts and implementation of this technology (Clemens et al. 2002).

One key area where our current knowledge is incomplete involves the mechanisms plants use to traffic and transport heavy metals from roots to above ground plant tissue. Fortunately, scientists have been able to shed light on important aspects of plant heavy metal trafficking and transport in recent years. Numerous classes of membrane transporters have been identified which have important roles in metal uptake and transport to various organelles, and important components of *in-planta* metal trafficking are now understood (Hall and Williams 2003; Clemens 2006b). Furthermore, the widespread use of tools from both genomics and molecular biology provide useful insights into the structural, mechanistic, and regulatory motifs present in numerous transport proteins, such as P-type ATPases (Kuhlbrandt 2004). Despite these advances, however, important questions involving heavy metal transport from roots to shoot have remained unanswered. Basically, these questions involve the following: (1) what are the xylem loading pathways, and (2) what metal binding ligands are used in long-distance xylem transport.

Some research has started to clarify questions in these two areas. Work in *Arabidopsis* suggested that AtHMA2 and AtHMA4 (members of the P_{1B} class of P-type ATPases) may be involved heavy metal xylem loading. This was based on fusion proteins using β-glucuronidase (GUS) as a marker that showed AtHMA2 and AtHMA4 localized to vascular tissue (Hussain et al. 2004; Verret et al. 2004). Furthermore, AtHMA4 knockout mutants were shown to be more sensitive to Cd (Hussain et al. 2004), and overexpression of AtHMA4 resulted in plants with higher shoot Cd and Zn content (Verret et al. 2004). Later work on AtHMA4 confirmed that *Arabidopsis* knockout mutants were more sensitive to Zn and Cd which led to the suggestion that AtHMA4 had dual roles in xylem loading and metal detoxification (Mills et al. 2005a).

Other research has focused on the roles of certain metal binding ligands used by plants. Since nearly all metal ions are expected to be ligand bound rather than exist freely in the cytoplasm (Clemens 2006b), understanding the role of these compounds appeared important for efforts directed towards engineering plants to accumulate high shoot metal levels. Phytochelatins (PCs) are the major type of heavy metal binding peptide which were first isolated in plants from cell suspension cultures and had the general structure $(\gamma\text{-glu-cys})_n\text{-gly}$ (n = 2–11)(Grill et al. 1985). These peptides were found to be non-translationally synthesized by phytochelatin synthase in response to a variety of metals (Grill et al. 1987; Grill et al. 1989). They also had a surprisingly wide distribution as they appeared to be universally present in higher plants and were found in representatives across all eukaryotic kingdoms (Clemens 2006a). Generally, once synthesized and bound to a heavy metal, PCs have been suggested to be primarily involved in shuttling the metal to the vacuole for storage and sequestration (Zenk 1996; Cobbett 2000; Clemens 2001).

Whether or not PCs have an additional role in long distance transport was still an open question with conflicting answers in the research literature, despite the importance of this issue to phytoremediation and food safety efforts. The relevant research and controversy over this potential additional role of PCs is outlined below. In one study, x-ray absorption spectroscopy was used to examine binding ligands for Cd in *Brassica juncea*, a plant with phytoremediation potential (Salt et al. 1995b). In roots and shoots, Cd was coordinated to four S ligands while xylem sap data indicated Cd coordination with six oxygen or nitrogen ligands. These results suggested that PCs had no significant role in long distance heavy metal transport, but were important for binding in root and shoot tissues. In a review of their own work (Salt et al. 2002), it was suggested that translocated Cd resulted from a couple of possibilities: either it never underwent sulfur coordination in roots and was therefore never stored, or it was

moved from storage to transport forms through an active process. Possible mechanisms behind this potential ligand switching were not understood.

However, later studies used tools from molecular biology and demonstrated that PCs were capable of long distance transport. Using the cad1-3 mutant of *Arabidopsis* (a PC synthase deficient line), TaPCS1 from wheat was transformed into this strain under the control of the alcohol dehydrogenase promoter (Adh::TaPCS1) which limited gene expression to root tissue (Gong et al. 2003). The root-specific protein expression was confirmed by RT-PCR as well as mRNA and protein blots, and transgenic plants did not display the metal-sensitive phenotype of cad1-3 plants. Furthermore, PCs were detected in the stem and leaf tissue of plants expressing Adh::TaPCS1 despite the absence of PCS protein in these tissues, which provided evidence for long distance root-to-shoot transport of PCs (Gong et al. 2003). Although overall PC levels were similar between WT controls and plants that expressed TaPCS1, transgenic lines contained more Cd in their stem and leaf tissue. The mechanism behind this phenomenon, however, was unclear. Later work by this same laboratory found evidence for long distance transport of PCs in the opposite direction (shoot-to-root) in *Arabidopsis* as well (Chen et al. 2006). Here, TaPCS1 was expressed in cad1-3 plants and was under control of the shoot-specific CAB1 promoter; validation of shoot specific expression consisted of northern blot and mRNA analysis. Although the transgenic plants were unable to rescue the root-sensitivity of cad1-3, PC_2 was clearly detected in root tissue by high performance liquid chromatography (HPLC) and mass spectrometry. Furthermore, grafting of a WT shoot onto a cad1-3 atpcs2-1 mutant (plants were lacking both the PCS1 and PCS2 genes) resulted in detection of PC_2, PC_3, and PC_4 in roots, which demonstrated that under their native promoter, PCs were also capable of shoot-to-root transport (Chen et al. 2006). Taken together, these papers provided strong, although indirect,

evidence for long distance PC transport in plants—or at least *Arabidopsis*—and therefore possible involvement of PCs in long distance metal transport.

Another study relied upon direct analysis of xylem sap in order to understand the potential role(s) of PCs. Using size exclusion HPLC coupled with a UV detector, fraction collector, and offline graphite furnace atomic absorption spectroscopy (GF-AAS), phytochelatins were detected in *B. juncea* xylem sap under Cd stressed conditions which consisted of a 50 μM Cd exposure for 10 h (Wei et al. 2007). The only other treatment was a 10 μM Cd exposure for 10 h, which did not apparently induce significant PC transport in the xylem sap. Unfortunately, no data was presented showing the limits of detection and quantitative reliability of their HPLC system and therefore PCs may have been present, but undetected in the 10 μM treatment. GF-AAS measurements on the collected fractions suggested that 29% of the xylem sap Cd was present as PC-Cd while the majority (68%) was bound to organic acids or inorganic ions (Wei et al. 2007). However, in metal speciation studies difficulties frequently arise with finding a sampling method that does not disrupt the native chemical equilibrium of the target of interest (Callahan et al. 2006). In the work of Salt et al. (1995b) and of Wei et al. (2007), it cannot be ruled out that sampling and preparation methods may have altered the chemical equilibria of the xylem sap.

Therefore, the role of PCs in long-distance transport has not yet been satisfactorily resolved. Plant molecular biologists have shown the capability of PCs to undergo long distance transport, but direct analysis of xylem sap has led to conflicting results. Furthermore, the concentration of Cd that was used to obtain a strong xylem sap response in the work of Wei et al. (2007) was high and outside of ranges likely to be found in the environment, even in contaminated sites. Thus, there is an open question regarding the role of PCs in long distance xylem transport in response to Cd

concentrations that are closer to what may be encountered at a contaminated site. Here, we present work that was focused into two areas: (1) measurement of PC_2 and other thiol containing compounds in response to Cd exposure in the xylem sap of *Brassica napus*, and (2) measurement of *B. napus* Cd uptake in a flowing culture system. The thiol work was focused on the temporal responses of xylem sap thiols to various Cd concentrations, and we provide further evidence for a possible role of PCs in long distance metal transport. The Cd depletion work builds upon previous Cd depletion efforts that were discussed in previous chapters. In this case, results are shown that indicate it was possible to differentiate between two fundamental mechanisms through which Cd interacts with plant roots in a flowing culture system: apoplastic binding and symplastic uptake.

5.2. Materials and methods

5.2.1. Plant growth and rearing

B. napus cv Quantum seeds were kept refrigerated at 4°C until needed, and were germinated in an aerated glass beaker placed in a controlled temperature water bath as previously described in chapter 3. *B. napus* plants were grown in a growth chamber in Ken Post Labs at Cornell University. Lighting was on a 16 h/8 h light/dark cycle, and chamber temperature was set at 23°C/20°C during the light and dark cycles, respectively. Plants were grown in the liquid culture rearing system as described in chapter 3, with the exception that the water filled heat sink was no longer needed as the growth chamber provided better environmental control and heat removal. Additionally, growth was staggered such that 8 plants were ready for use each week and plants were randomly placed within the rearing unit in order to

eliminate any potential effects of location within the rearing unit on experimental results. Nutrient solution was a modified Johnson's solution (Johnson et al. 1957) and had the composition indicated in Table 3.1 in chapter 3. Plants were grown in the rearing system starting with day 5 of the growing schedule and remained in this unit until days 28-30, depending on the experiment that was being conducted.

5.2.2. Experimental trough system

The trough system used in these experiments was an adaptation and simplification of ECUDS, which was fully described in chapter 3. A diagram of this simplified design is shown in Figure 5.1. These design changes were necessitated by the time scales over which the xylem collection experiments were conducted as well as by experimental throughput considerations that dictated using all of the available plants in a given growth cycle. Briefly, the simplified system used two troughs and the solenoid valve system as well as the ISE data acquisition system of the original design was not used in this configuration. A new stand was constructed to accommodate up to three troughs and allowed troughs to be placed side-by-side in the growth chamber. Fluid for each trough was kept in separate reservoirs, and was pumped by a Masterflex LS digital drive peristaltic pump that was equipped with two pump heads (Masterflex Easyload 3, Cole-Parmer, Inc.). Flow rate was set at 200 mL/min and total system volume for each trough in these experiments was 10 L, which resulted in a mean volumetric retention time of 50 min. The system was generally operated in recirculation mode, where trough effluent reentered the storage reservoir via gravity flow and was then pumped from this reservoir back into the trough.

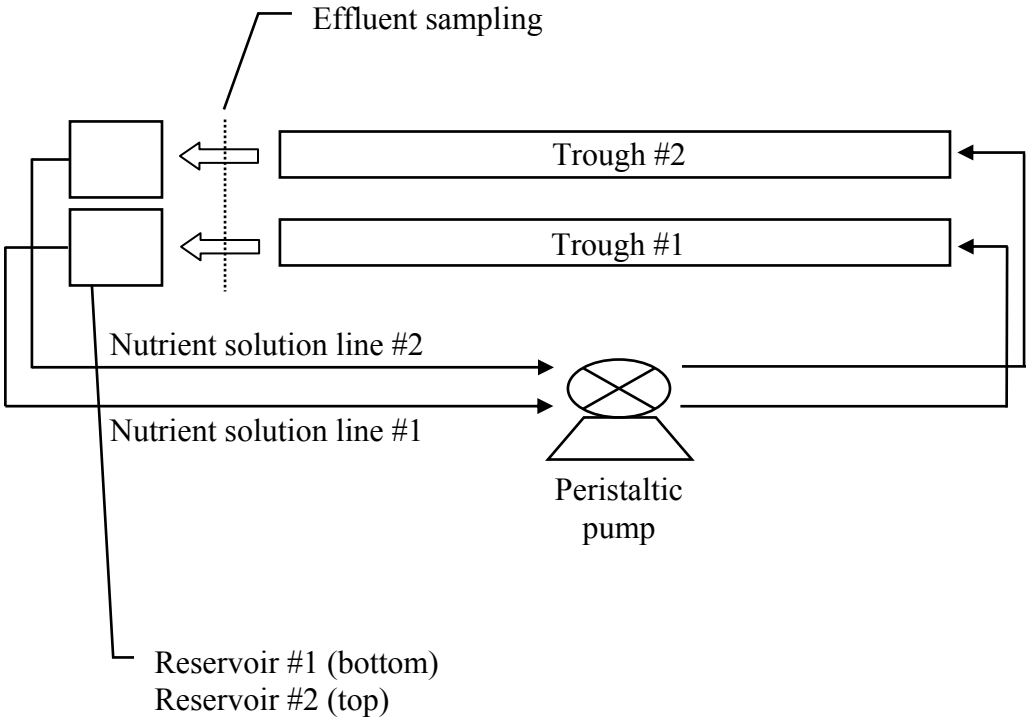

Figure 5.1. Diagram of the simplified ECUDS configuration that was used for the xylem sap and Cd uptake experiments. Each reservoir held 10 L of nutrient solution, and arrows indicate the direction of fluid flow in the system.

5.2.3. Cd depletion and xylem sap collection experiments

On days 28-30 (depending upon the desired Cd exposure time) plants were removed from the rearing unit and placed into the trough system. Each trough received a distinct treatment and 4 plants were used in each trough per experiment. Therefore, all plants within a trough shared the same Cd treatment. In order to minimize the effects of transport and handling shock, *B. napus* plants were always allowed to equilibrate in the trough system for 24 h with a nutrient solution that was lacking Fe-HEDTA and Cu prior to Cd treatment. After equilibration, plants were exposed to a Cd solution for one of three time periods: 24 h, 48 h, or 72 h, depending on the experiment. After this period, plant stems were cut and xylem sap collection was initiated; collection experiments were designed so that they always occurred on day 32 of a growth cycle. For these experiments, the tested Cd concentrations were 0.5 μM, 1 μM, and 5 μM.

Xylem sap was collected by detopping each plant just below the cotyledon internode using a fresh razor blade (VWR International). New razor blades were used for each plant in order to ensure sharpness and avoid any potential cross-contamination between plants. Immediately after detopping, the stem was washed for two minutes under a constant stream of 18 MΩ deionized water. Then, depending upon stem size, a 1 ½ in. length of tubing (3/16 in. or ¼ in I.D.,VWR International.) was fitted to the tops of the stems. Silicone grease (Radiator Specialty Products, Inc.) was used to seal the base of the tubing on the stem to avoid potential leakage. Xylem sap was then allowed to exude under natural root pressure, and was collected at hourly intervals for 3 h. Sap was collected by pipette into 1.5 mL plastic tubes, which were kept on ice during the collection period. After collection, a small amount (5-30 μL, depending upon sap volume) of 800 mM methanesulfonic acid (MSA, Sigma-Aldrich,

Inc.) was added to each tube so that the final MSA concentration was approximately 10 mM in order to denature proteins and preserve thiols. Samples were then stored in a -20°C freezer until HPLC analysis, which was conducted within 72 h of collection.

Additional experiments were conducted to investigate plant-free behavior of the trough system by recirculating Cd nutrient solution in a trough that was lacking any plant material. These experiments were conducted over a period of 72 h in order to mimic the time frame of the longest Cd exposure periods. Other experiments involved studying the Cd adsorption characteristics of dead *B. napus* roots. These studies were conducted by excising the root mass of four plants aged 29 days and immersing them in boiling DI water for 3 min in order to eliminate symplastic uptake. After immersion, roots were blotted to dryness on paper towels to remove excess water and then placed in the hydroponics trough. Once roots from all four plants were in the trough, system solution was changed to one containing Cd, and depletion measurements were initiated. Root adsorption experiments used Cd concentrations of 1 μM, 5 μM, and 10 μM.

In all experiments, Cd depletion in troughs was monitored by pipetting 10 mL samples of the trough effluent into 15 mL disposable plastic tubes (VWR International.). Samples were taken every 5 min for the first 30 min followed by a sample at 45 min, 1 h, and then hourly thereafter for a period of 5-7 h. Additional samples were taken at longer time periods as appropriate for the experiment being conducted. After collection, 0.5 mL of HNO_3 (EMD Chemicals, Omnitrace purity) was added within a few hours of acquiring each sample to avoid Cd adsorption to the container surface, and each sample was then syringe filtered through a 0.2 μm, 16 mm diameter nylon filter (National Scientific). Cd concentration determinations were made using ICP-AES (Thermo Jarrel Ash ICAP-60) that had previously undergone extensive modifications (Rutzke 2002). In order to obtain the root mass that was

present in each experiment, roots were collected, dried in a 100°C oven for 72 h, and then weighed. All root mass reported here is on a dry weight basis.

5.2.4. Xylem sap thiol measurements

Xylem sap thiols were analyzed by largely following a previously published method (Wei et al. 2003) with some slight alterations. Briefly, this method involved labeling thiols with monobromobimane (mBrB) followed by separation and quantification on an HPLC (Kosower et al. 1979; Newton et al. 1981; Ahner et al. 1995). Borate-DTPA buffer was added to samples followed by the addition of DTT in order to reduce disulfide bonds. Samples were then reacted with mBrB according to the method of Newton et al. (1981). MSA was added at the end of the reaction to stabilize the fluorescently tagged thiols. Chromatography was carried out on a Shimadzu HPLC equipped with a fluorescence detector (RF-10AXL) set to an excitation wavelength of 390 nm and an emission wavelength of 478 nm. The C_{16} column (Supelco Discovery RP Amide C_{16}) was kept at 25°C in an oven (Prominence CTO-20AC) during all HPLC runs. The mobile phases consisted of HPLC grade acetonitrile (Mallinckrodt Chemicals ChromAR) and 25 mM KH_2PO_4/H_3PO_4 buffer at pH 3.55. A lower pH buffer was used in Wei et al. (2003), but buffer at that pH did not sufficiently resolve all thiol groups of interest on our system. The gradient time program that was used is shown in Table 5.1. This method was capable of simultaneously resolving four distinct thiols: cysteine (J.T. Baker, Inc.), glutathione (GSH, Sigma-Aldrich, Inc.), γ-glutamyl cysteine (γ-EC, Sigma Aldrich, Inc.), and phytochelatin (n = 2, Cell Essentials, Inc.).

Table 5.1. HPLC gradient program

Time (min)	% Acetonitrile
0	8
4	8
14	13
17	16
27	18
33	40
36	80
40	80
42	8
47	8

5.2.5. Data analysis

Thiol concentration results from the HPLC were averaged across replicates. In order to determine differences in mean concentration values between xylem sap thiols within a treatment group, ANOVA model I (the cell means model) was used:

$$Y_{ij} = \mu_i + \varepsilon_{ij} \quad \text{[Eq. 5.1]}$$

where:

Y_{ij} = value of response variable in the j^{th} trial and i^{th} factor level;

μ_i = mean of factor level i;

ε_{ij} = error term, $N(0, \sigma^2)$

The factor, Cd concentration, was considered a fixed effect in this model, and treatment groups consisted of plants that were exposed to Cd for the same period of time—i.e. thiol concentration results were grouped according to Cd exposure time. Therefore, the ANOVA model tested for differences among xylem sap thiols across plants that shared the same Cd exposure period but were exposed to different Cd concentrations. MINITAB 15 (Minitab, Inc.) was used for all ANOVA computations which used the general linear model (GLM) in order to properly account for unbalanced data. The GLM analysis option used the same basic framework for analysis (Eq. 5.1) with the exception that error calculations involved using the appropriate number of replicates per cell rather than assuming equal replicates across cells as in a balanced design. In all cases, statistical tests were formulated with a null hypothesis (H_0) of no difference(s) among treatment group means and an alternative hypothesis (H_A) of difference(s) among treatment group means. Results were considered statistically significant at the $\alpha = 0.05$ level. Further analysis was done among treatment means by using the Tukey-Kramer multiple comparison procedure, which looked for differences among all pairs of means within a treatment group. In

these multiple comparisons, the family Type I error rate was controlled at $\alpha = 0.05$. Some aspects of trough Cd concentration profiles were mathematically modeled using the following two-parameter model:

$$y = \beta_1 + \beta_2 t \qquad \text{[Eq. 5.2]}$$

where:

y = Cd concentration (mole L^{-1});

t = time (hours);

β_1, β_2 = model parameters, with units of (mole L^{-1}) and (mole)(L^{-1} h^{-1}), respectively

Eq. 5.2 is linear in the parameters and therefore was fit to the data using linear least squares in KaleidaGraph 3.6 (Synergy Software) to generate least squares estimates of the parameters. Model assumptions were further checked using MINITAB 15 to ensure validity of statistical inferences.

5.3. Results

5.3.1. Cd depletion measurement results

Understanding the behavior of Cd within the simplified version of ECUDS was an important part in identifying plant-specific interactions with Cd in this system. Therefore, experiments were designed to examine system Cd behavior under two different scenarios: one with no plant material in the system, and one with dead *B. napus* roots. Although earlier experiments that were conducted in ECUDS suggested that Cd interactions with trough components were negligible, system design had changed and therefore these system characteristics required reevaluation. Results from a 74 h constant concentration, plant-free experiment are shown in Figure 5.2.

Eq. 5.2 was fit to this data using linear least squares; this analysis indicated that time was not a statistically significant predictor variable ($p = 0.12$) and that fitting a constant term adequately described the data ($p < 0.0001$). Therefore, because system Cd concentration did not change over time in a plant-free setting, Cd interactions with the trough as well as evaporative losses were considered negligible, and it was concluded that these processes were not capable of causing measurable changes in system Cd concentration.

After establishing this baseline characteristic, experiments were conducted using roots that had been previously killed by a brief immersion in boiling DI water. With the exception of using dead roots, all other system parameters remained the same. The Cd concentration profiles for these dead root experiments are shown in Figure 5.3(a-b), which shows results from experiments that used initial Cd concentrations of 10 μM, 5 μM, and 1 μM. From these profiles, a period of rapid depletion immediately occurred at the beginning of each experiment which is clearly depicted in Figure 5.3b. The initial decline in system concentration was consistent with rapid Cd binding to the root apoplast that quickly reached equilibrium, which then allowed system solution to thoroughly mix and stabilize the Cd concentration by about 45 min. After this period, system concentrations underwent relatively little change through 24 h, and then tended to increase in subsequent time periods, particularly at the 5 μM and 10 μM Cd concentrations. This concentration increase was consistent with a hypothesis of binding site degradation in the heat killed roots as time progressed. Concentration stability was further examined by fitting Eq. 5.2 to the data between 1 and 24 h to see if time was a significant predictor variable of Cd concentration. Results of these linear regressions are shown in Table 5.2. At all

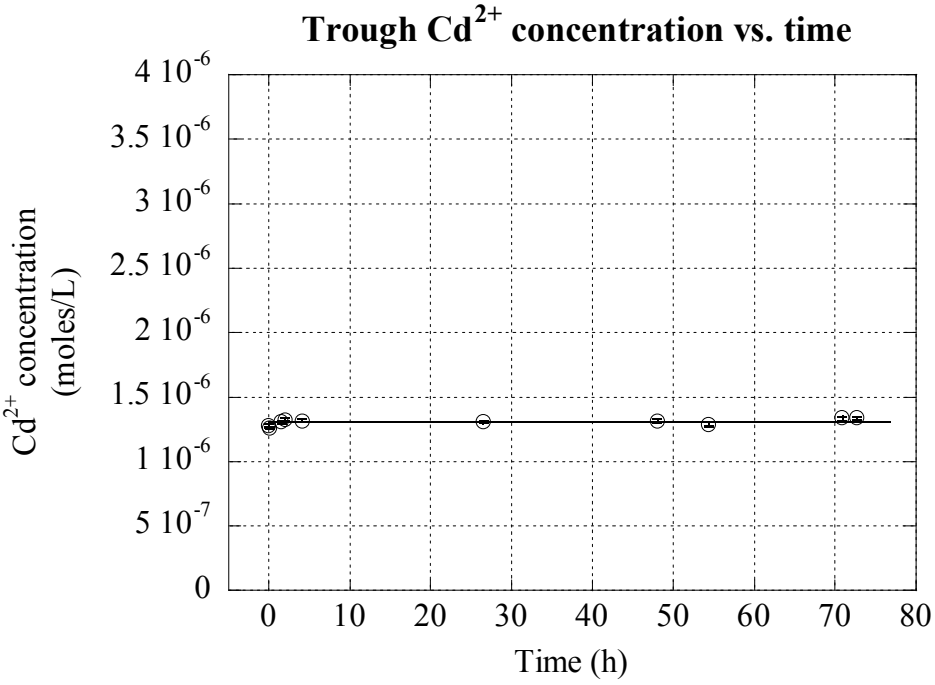

Figure 5.2. Trough Cd concentration vs. time under plant-free conditions. Initial concentration was 1.3 μM Cd. Error bars represent the standard deviation reported from the ICP. These errors were small, and obscured by the graph symbols.

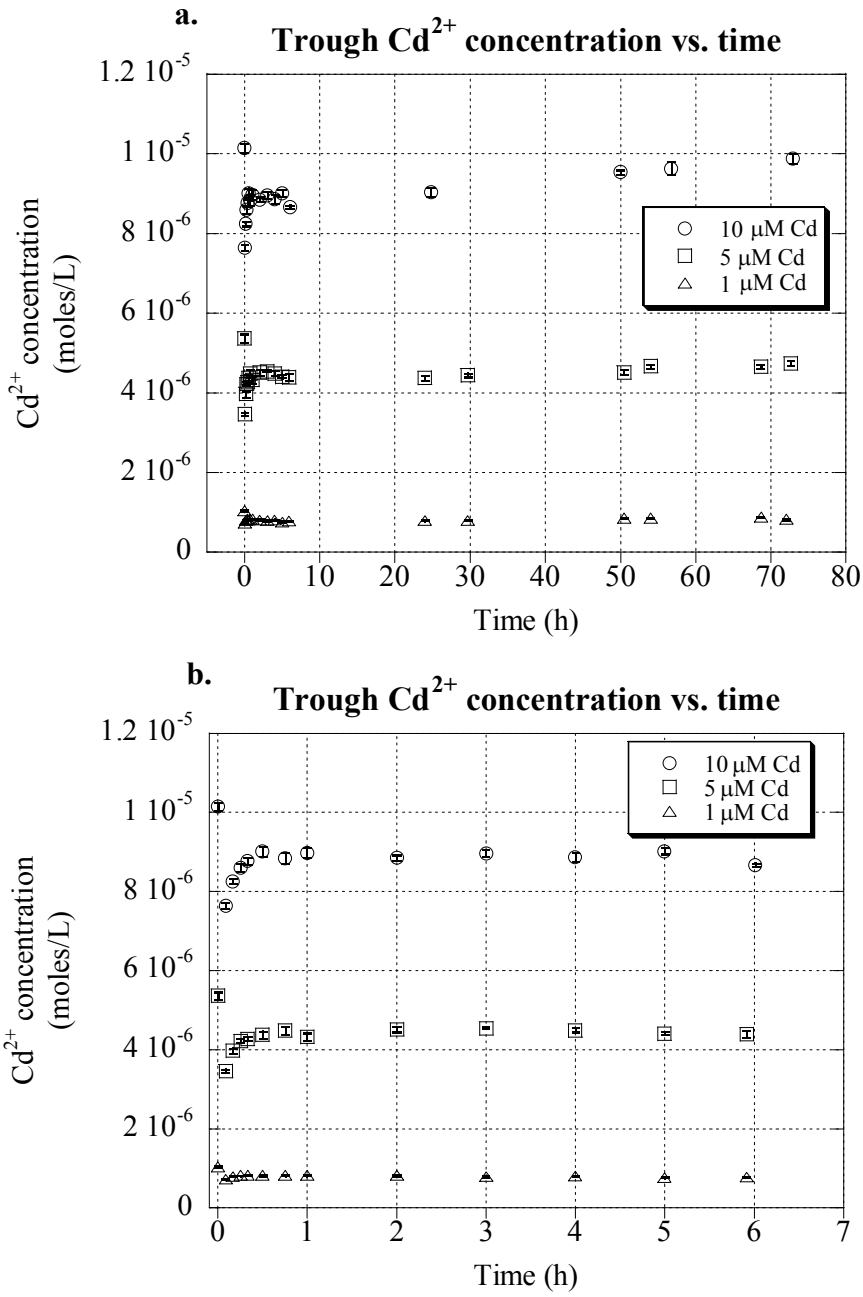

Figure 5.3. Trough Cd concentration vs. time over (a) 72 h and (b) 6 h for experiments using dead *B. napus* roots. Error bars represent the standard deviation reported from the ICP.

Table 5.2. Regression results for 1-24 h data from trough experiments using dead *B. napus* roots,.

Cd^{2+} concentration	Equation	R^2	Predictor *p*-value
10 μM	$y = 8.9\times10^{-6} + \left(5.7\times10^{-9}\right)\ t$	0.13	0.43
5 μM	$y = 4.5\times10^{-6} - \left(3.5\times10^{-9}\right)\ t$	0.12	0.45
1 μM	$y = 8.0\times10^{-7} - \left(4.3\times10^{-10}\right)\ t$	0.02	0.75

concentrations, the linear model was not statistically significant (p-values < .05) and the resulting model slopes had small absolute values. Therefore, time was not a good predictor variable of Cd concentration in the trough system with dead roots between 1 h and 24 h, which indicated that after an initial period of binding, concentration did not significantly change through 24 h. To quantitatively assess total bound Cd, concentration data between 1-24 h was averaged and converted to moles Cd by multiplying the concentration by total system volume. This value was then subtracted from the initial moles of Cd in the system and divided by root dry weight in order to normalize adsorbed Cd to root mass. Results of these calculations are shown in Figure 5.4, where total adsorbed Cd is shown to increase in response to higher initial Cd concentrations. These values are higher than the adsorbed Cd values shown in Figure 4.5 in chapter 4 due to the differences in total volume between the batch and trough systems. Because of these volume differences, the amount of Cd present in each system was different, and thus the total amount of adsorbed Cd was somewhat different between the systems. Overall extent of Cd binding was also calculated for each experiment and is shown for the first 24 h at each concentration in Figure 5.5(a-c). Here, extent of binding is shown to have an initial spike to between 0.24-0.35, depending upon the experiment, which then stabilized to between 0.11 and 0.20 and remained near these values through 24 h. Taken together, these results demonstrated that nonspecific Cd binding to the root apoplast occurred rapidly (< 1 h), was constant through at least 24 h, and resulted in system Cd depletion of around 10-20%, depending upon initial Cd concentration.

Cd depletion results from a representative experiment using live *B. napus* plants in the trough system are shown in Figure 5.6(a-b). The initial Cd concentration was 1 μM. In Figure 5.6a, Cd depletion measurements over the entire experiment are shown. Data from this figure demonstrate that system Cd was depleted within 24 h of

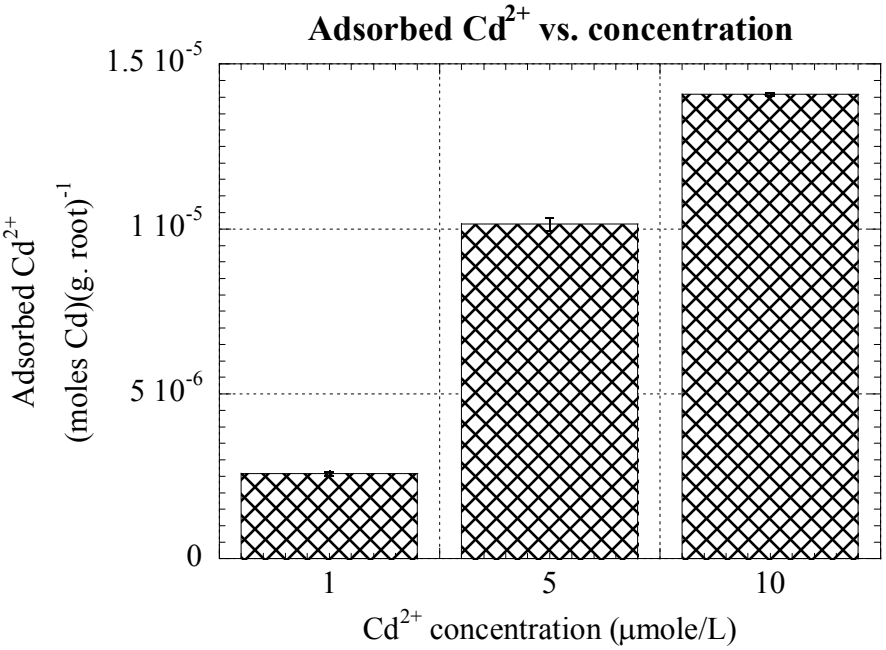

Figure 5.4. Moles of adsorbed Cd vs. initial trough Cd concentration for the dead root experiments. Adsorbed Cd values were normalized to *B. napus* root mass. Error bars represent the standard deviation of the mean.

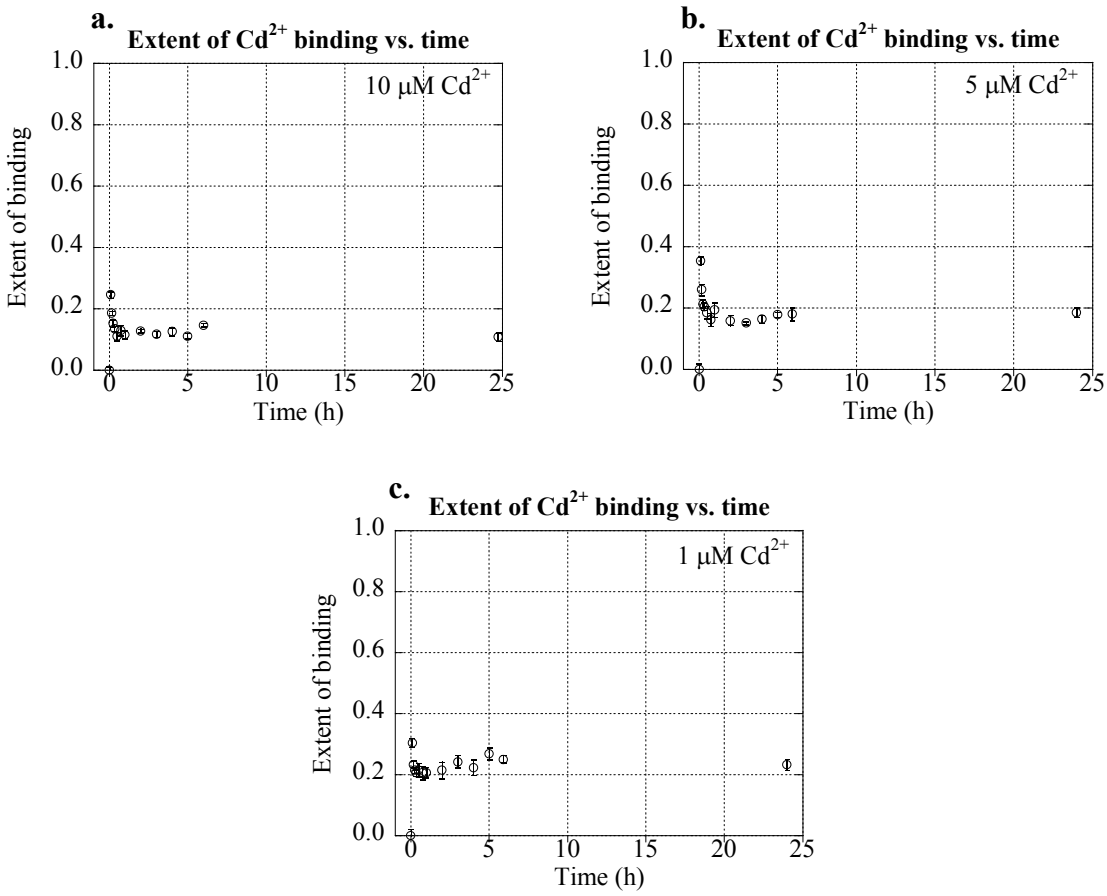

Figure 5.5. Extent of binding for the first 24 h for the (a) 10 μM, (b) 5 μM, and (c) 1 μM Cd experiments conducted with heat killed roots of *B. napus*. Error bars represent the error information reported by the ICP.

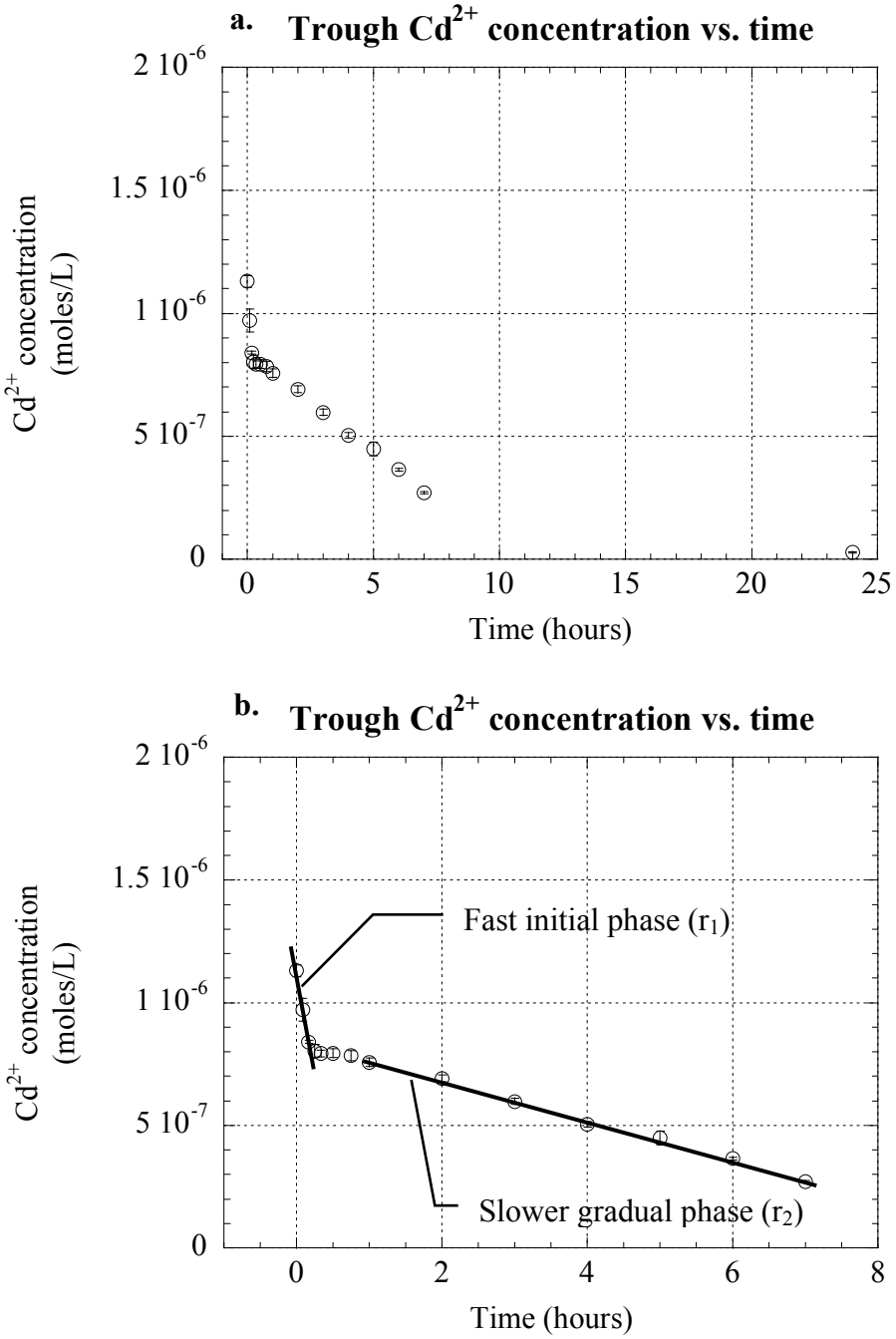

Figure 5.6. Trough Cd concentration vs. time for a representative experiment using live *B. napus* plants during (a) the entire experiment, and (b) the first 7 h. Biphasic depletion kinetics are highlighted in (b). Error bars represent error information reported from the ICP.

experiment initiation. In fact, the measured Cd concentration at 24 h was either at or below the detection limit of the ICP, and for this reason the 24 h data point was viewed as an instrument artifact rather than an accurate representation of system Cd concentration at that time point. Another important trend shown in this figure is the presence of biphasic depletion kinetics. Figure 5.6b more clearly illustrates this phenomenon: a period of rapid initial depletion occurred during the first 20 min (r_1), after which Cd concentration stabilized and then underwent a longer period of gradual decline between hours 1-7 (r_2). When compared to the plant-free concentration data in Figure 5.2 and the dead root data in Figure 5.3, some observations are worth noting. First, both the dead root and live plant data indicated a rapid initial decline in trough Cd concentration. Second, after this period of initial decline, Cd concentration stabilized. Third, a period of gradual Cd depletion occurred during hours 1-7 in the live plant experiment that was not evident in either the plant-free or dead root experiments. These trends were observed in all other experiments that used live roots (data not shown), and therefore live roots interacted in a repeatable and specific way with Cd.

The two depletion rates were further characterized for all dead and live-root experiments. To quantify the initial depletion rate (r_1), linear least squares were used to fit Eq. 5.2 to the first two or three data points in each experiment. The slope of the resulting line (β_2) was then multiplied by the system volume and divided by the root mass to convert the value to units of (moles Cd)(h)$^{-1}$(g. root)$^{-1}$. The gradual depletion rate (r_2) was calculated using the same method, with the exception that only data after the first hour was used. In this case, between 4-7 data points were used to fit Eq. 5.2, depending upon how rapidly Cd depletion reached the detection limit of the ICP. These normalized rates are designated as r_1' and r_2'. Results of these rate calculations are shown in Table 5.3, along with R^2 and p-values for the model fit to r_2 data. In all

Table 5.3. Calculated uptake rates from trough Cd depletion data

Cd concentration	Time (h)	r_1' (moles Cd) $(g.\ root)^{-1}(h)^{-1}$	r_2' (moles Cd) $(g.\ root)^{-1}(h)^{-1}$	R^2	Model *p*-value
5 μM	24	1.3×10^{-5}	7.3×10^{-7}	0.95	<.001
	48	1.4×10^{-5}	7.9×10^{-7}	0.99	<.001
	72	4.0×10^{-5}	7.2×10^{-7}	0.99	<.001
1 μM	24	1.3×10^{-5}	5.9×10^{-7}	0.99	<.001
	48	2.7×10^{-5}	6.6×10^{-7}	0.99	<.001
	72	3.3×10^{-5}	5.2×10^{-7}	0.99	.003
0.5 μM	24	9.2×10^{-6}	3.3×10^{-7}	0.99	.003
	48	5.9×10^{-6}	1.5×10^{-7}	0.90	.013
	72	5.0×10^{-6}	1.2×10^{-7}	0.99	<.001
Dead roots					
10 μM	72	3.4×10^{-4}			
5 μM	72	2.5×10^{-4}			
1 μM	72	4.0×10^{-5}			

cases, r_1' was greater than r_2' by factors ranging from 17-63. Model fits to r_2 data were all statistically significant (p-value < 0.05) and exhibited high R^2 values, which suggested that Eq. 5.2 adequately described temporal trends in Cd concentration data during the gradual phase of depletion. Additionally, the 5 μM and 1 μM dead root experiments exhibited higher r_1' values when compared to live root experiments at the same concentration. Further comparisons of the original rates, r_1 and r_2, were done by calculating half-depletion times using the following formula:

$$t_{1/2} = \frac{\left(Initial\ moles\ Cd/2\right)}{V\left(r_x\right)} \qquad \text{[Eq. 5.3]}$$

where:

V = system volume

r_x = r_1 or r_2

Values from Eq. 5.3 were averaged across a concentration group and the results are shown in Table 5.4. From this table, half-depletion times for r_1 were all under 1 h while half depletion times for r_2 ranged between 5-18 h. Clearly, the high rate of r_1 was not maintained for very long since it would have resulted in extremely rapid depletion to below detectable limits. Therefore, different mechanisms were likely responsible for the two different phases of Cd depletion.

Plots of r_1' and r_2' versus initial Cd concentration are shown in Figure 5.7(a-b). In the case of r_1', very large error values are present, which may have obscured a response in this rate to initial concentration. An increase in r_1' may have been present as Cd concentration increased from 0.5 μM to 1 μM, but large error values made it difficult to draw many conclusions from this data. In contrast, however, the plot of r_2' vs. initial concentration in Figure 5.7b shows reduced error and a more easily characterized response to initial Cd concentration. Here, r_2' increased in value as Cd concentration rose, but the largest increase was between 0.5 μM and 1 μM Cd, with

Table 5.4. Calculated half-depletion times for r_1 and r_2

Cd concentration	$t_{1/2}$ of r_1 (h)	COV	$t_{1/2}$ of r_2 (h)	COV
5 μM	0.870	0.692	17.6	0.366
1 μM	0.206	0.507	7.30	0.156
0.5 μM	0.143	0.0341	5.37	0.400
Dead root, 10 μM	0.169			
Dead root, 5 μM	0.113			
Dead root, 1 μM	0.134			

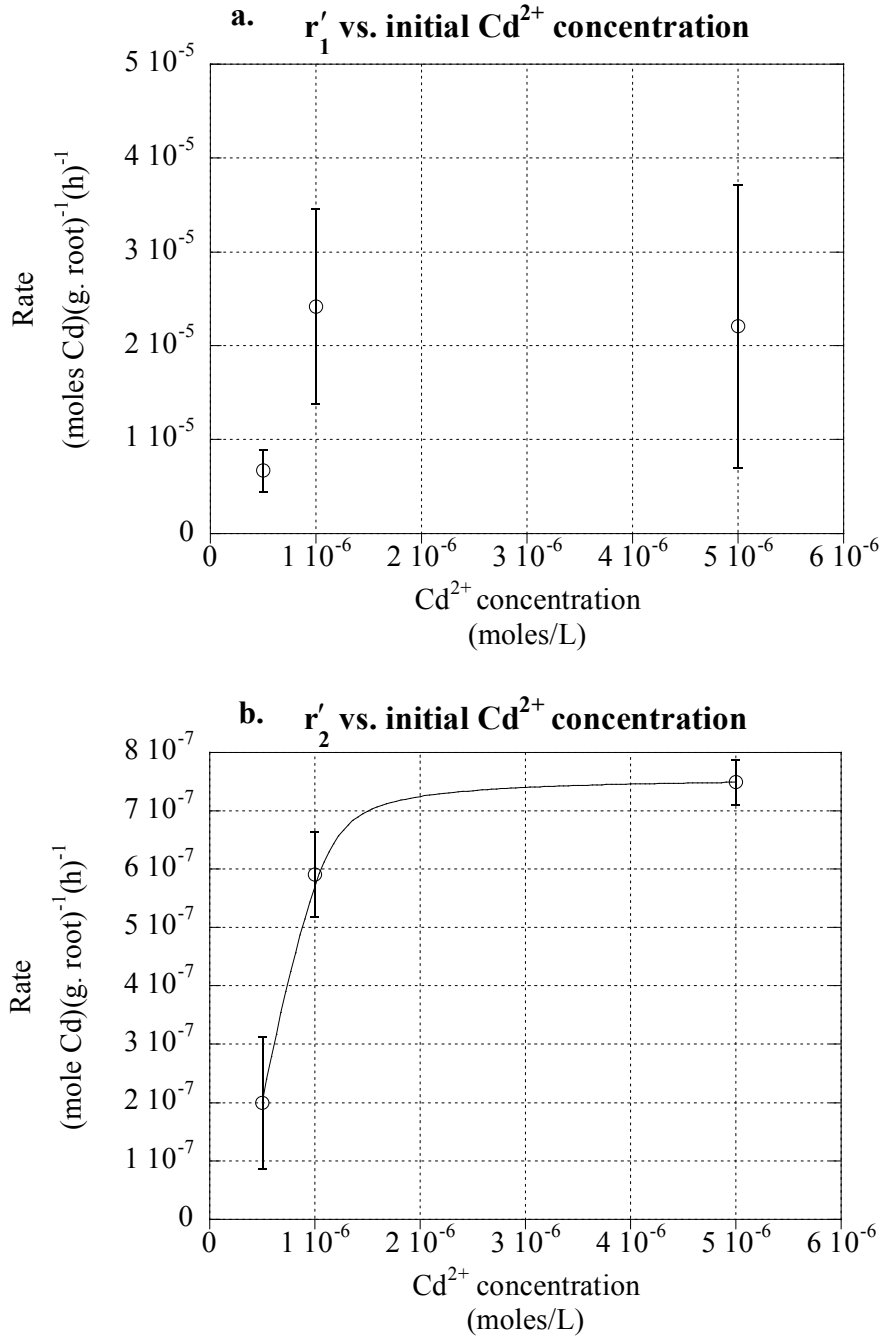

Figure 5.7. Plots of (a) r_1' and (b) r_2' vs. initial Cd concentration in the trough system. Error bars represent the standard deviation of the mean.

less of a response observed in r_2' between 1 μM and 5 μM Cd. In both cases, plots of r_1' and r_2' versus initial Cd concentration suggested that saturable kinetics may have been present, and therefore both sets of data were fit to the Michaelis-Menten kinetic equation:

$$V = V_{max} \frac{C}{K_m + C} \qquad \text{[Eq. 5.4]}$$

where:

V = Cd uptake velocity (moles)(g. root)$^{-1}$(h)$^{-1}$

V_{max} = maximal Cd uptake velocity (moles)(g. root)$^{-1}$(h)$^{-1}$

C = bulk Cd concentration (mole L^{-1})

K_m = kinetic constant (moles L^{-1})

The Michaelis-Menten equation is nonlinear in the parameters, and therefore parameter values were calculated using nonlinear least squares in MATLAB 7.5.0 (The MathWorks, Inc.). Results from fitting Eq. 5.4 to both sets of data are shown in Table 5.5. In the case of r_1', a relatively low R^2 value and high SSE suggested a poor fit and therefore, Eq. 5.4 may not be an appropriate model for this data. Again, large error values hindered drawing conclusions. In contrast, Eq. 5.4 appeared to be a more appropriate model for describing trends in Figure 5.7b, as an R^2 value of 0.92 indicated that the Michaelis-Menten kinetic equation described a large amount of the variability in this data.

5.3.2. Xylem sap thiol measurement results

The thiol derivitization protocol adapted from Wei et al. (2003) provided a very sensitive method for detection of thiols labeled with mBrB. This method allowed for detection of cysteine, γ-EC, glutathione, and phytochelatin (n = 2) in amounts

Table 5.5. Results from fitting Eq. 5.4 to data in Figures 5.7a and 5.7b

Rate data	K_m	V_{max}	R^2	SSE
r_1'	5.66×10^{-7}	2.59×10^{-5}	0.42	1.1×10^{-9}
r_2'	6.35×10^{-7}	8.58×10^{-7}	0.92	8.8×10^{-14}

down to 5 pmole in a 100 μL volume. Higher order phytochelatins, however, were not resolvable using this technique. A HPLC fluorescence chromatogram of a xylem sap sample is shown in Figure 5.8. Xylem sap cysteine results across three time periods and three Cd concentrations are shown in Figure 5.9(a-b). Across the tested Cd concentrations, elevated cysteine levels were observed during the 48 and 72 hour time periods. Within the 24 hour treatment group, all means appeared equivalent and the *F* test failed to reject the null hypothesis, H_0, ($p = 0.64$). In the 48 hour exposure group, ANOVA *F* test results rejected H_0 of no significant differences among the Cd levels in favor of H_A. Tukey's multiple comparison procedure revealed significant differences between the control and 1 μM Cd treatments ($p = 0.0468$) as well as between the control and 5 μM Cd treatments ($p = 0.001$). Statistically significant differences were also found between the 0.5 μM Cd vs. 5 μM Cd treatments ($p = 0.003$) and 1 μM Cd vs. 5 μM Cd treatments ($p = 0.0172$). For the 72 h group, an additional step was required since the data were not homoskedastic. Here, data were log-transformed prior to statistical analysis in order to stabilize the variance and allow for ANOVA model assumptions to be met. The *F* test rejected H_0 in favor of H_A ($p < 0.001$). As seen in Figure 5.9a, all 72 h Cd treatments exhibited statistically significant differences from the control with *p*-values of 0.0357, 0.002, and 0.001 for the 0.5 μM, 1 μM, and 5 μM Cd treatments, respectively. Additionally, significant differences were found between the 0.5 μM vs. 1 μM Cd ($p = 0.0126$) and 0.5 μM Cd vs. 5 μM Cd treatments ($p = 0.0041$). No significant difference was found between the 1 μM and 5 μM Cd treatments ($p = 0.7320$).

Results reported in Figure 5.9a were re-plotted as a scatterplot in Figure 5.9b to better illustrate the temporal behavior of xylem sap cysteine concentration in response to Cd exposure. From this plot, two observations were noted: (1) the presence of detectable cysteine in the xylem sap of unexposed control plants suggested a basal

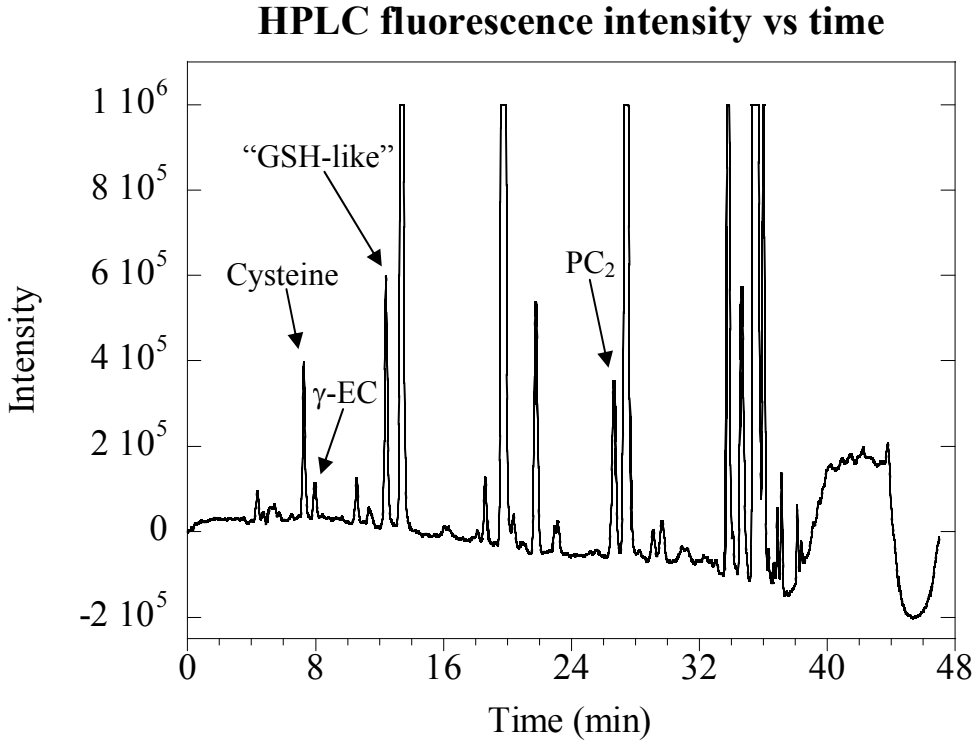

Figure 5.8. HPLC chromatogram of a xylem sap sample containing cysteine, γ-EC, "GSH-like", and PC_2.

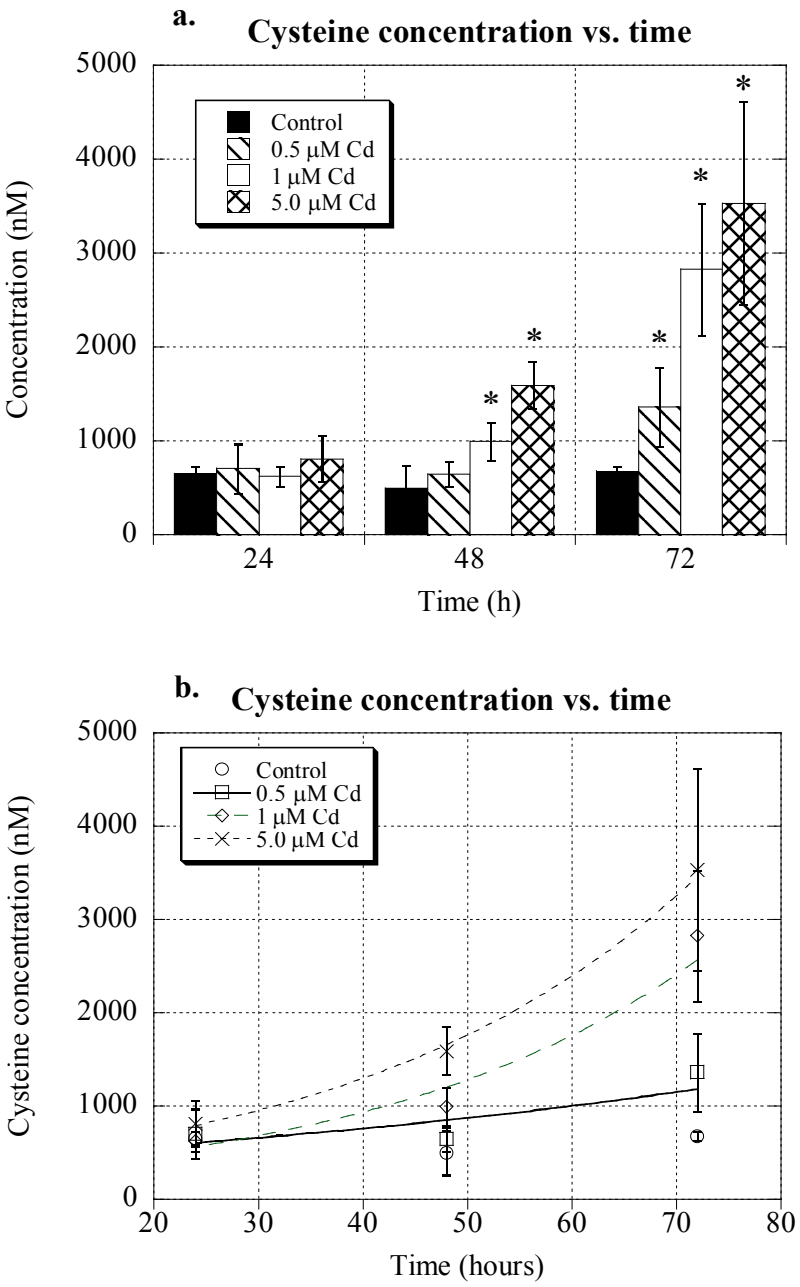

Figure 5.9 (a). *B. napus* xylem sap cysteine concentration vs. time. Within the three exposure periods (24, 48, and 72 h), an asterisk (*) indicates a statistically significant difference from the control at that time point. (b). Cysteine concentration vs. time data re-plotted as a scatterplot with Eq. 5.6 fit to the data. In both graphs, error bars represent the standard deviation of the mean.

level of cysteine was normally present, and (2) the rate of cysteine concentration change within a Cd exposure group appeared to increase over time. In other words, when looking at cysteine concentration vs. time, the slope was greater in magnitude between 48 and 72 h than between 24 and 48 h. This observation suggested that the kinetics might be first order in cysteine concentration as follows:

$$\frac{dC_{Cys}}{dt} = kC_{Cys} \qquad \text{[Eq. 5.5]}$$

where:

k = kinetic constant $(h)^{-1}$

C_{Cys} = cysteine concentration (nM)

Eq. 5.5 was then integrated into the following form:

$$C^*_{Cys} = C_1 e^{kt} \qquad \text{[Eq. 5.6]}$$

where:

C_1 = initial cysteine concentration (nM)

C^*_{Cys} = xylem sap cysteine concentration at time t (nM)

Eq. 5.6 was fit to the cysteine data using KaleidaGraph 3.6 (Synergy Software). Results from the fit are presented in Table 5.6. These results show that the integrated first-order rate expression (Eq. 5.6) fit the cysteine concentration data well in the 1 μM and 5 μM Cd experiments, where high R^2 values indicated that most of the variability in the data was explained by this model. At the lowest Cd exposure level, 0.5 μM, this model was less adequate at explaining trends in the data ($R^2 = 0.77$). A linear model (Eq. 5.2) did not result in a better fit than Eq. 5.6 (data not shown).

Results showing xylem sap phytochelatin concentration are shown in Figure 5.10(a-b). In this case, phytochelatin was detected at all tested Cd concentrations and at all time periods, but was never detected in unexposed control plants. In order to satisfy model assumptions for phytochelatin data analysis, data from the 24 h and 72 h

Table 5.6. Results from fitting Eq. 5.6 to the xylem sap cysteine data

Cd concentration (μM)	C_1 (nM)	k $(h)^{-1}$	R^2
5	379	0.0307	0.99
1	264	0.0316	0.98
0.5	438	0.0138	0.77

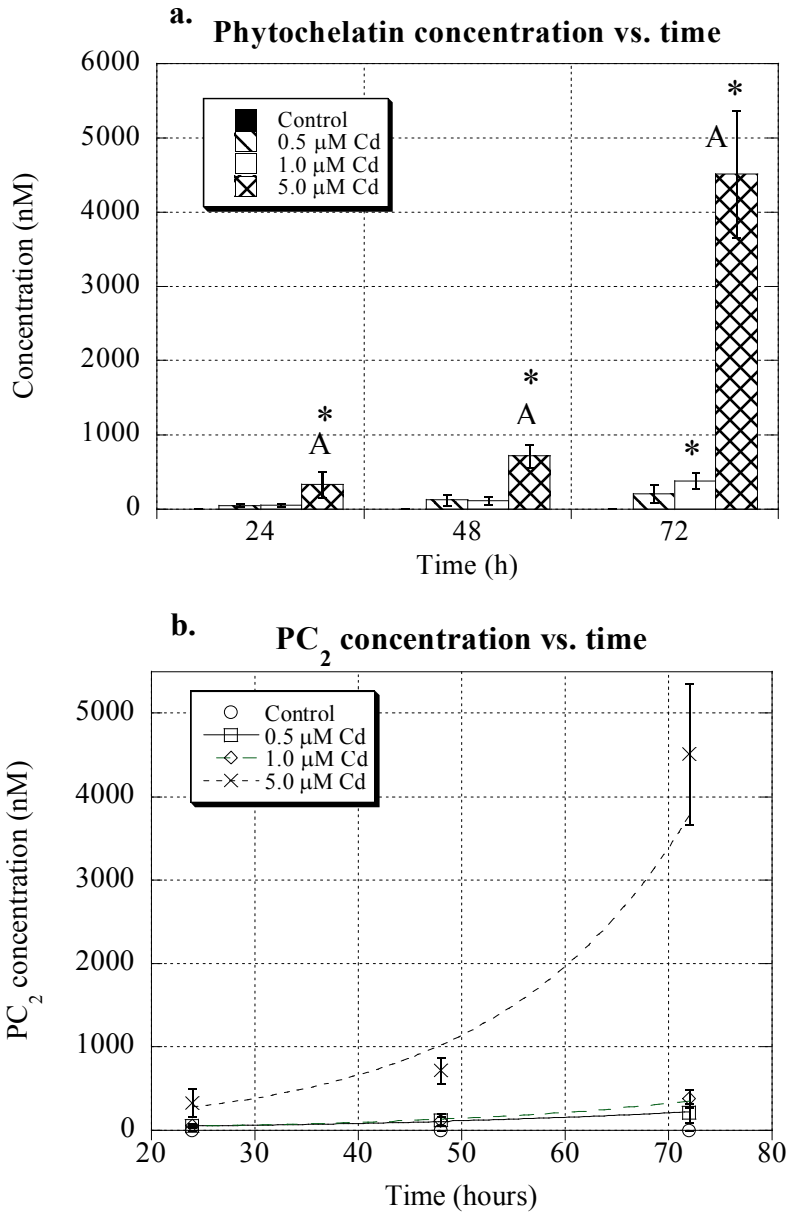

Figure 5.10 (a). *B. napus* xylem sap PC_2 concentration vs. time. Within each time period, an asterisk (*) indicates a statistically significant difference from the 0.5 µM Cd treatment, while statistically significant differences from the 1µM Cd treatment are indicated by the letter "A." (b). PC_2 concentration data re-plotted as a scatterplot with Eq. 5.6 fit to the data. In both graphs, error bars represent the standard deviation of the mean.

time periods was log-transformed, which stabilized data variances and allowed for homoskedastic model assumptions to be met. ANOVA *F* test results rejected H_0 in favor of H_A at all three time points with $p = 0.005$, $p < 0.001$, and $p < 0.001$ for the 24 h, 48 h, and 72 h treatment groups, respectively. Within the 24 hour group, xylem sap from the 5 μM Cd treatment was statistically different from the 0.5 μM and 1 μM treatments ($p = 0.0064$ and $p = 0.0092$). The 48 h group also showed significant differences between the 5 μM treatment and other concentrations with *p*-values of 0.0006 and 0.0017 when compared to the 0.5 μM and 1 μM Cd treatments, respectively. In the 72 h group, statistically significant differences existed between the following: 0.5 μM vs. 1 μM Cd ($p = 0.0012$), 0.5 μM vs. 5 μM Cd ($p < 0.0001$), and 1 μM vs. 5 μM Cd ($p < 0.0001$). When PC_2 concentration data was re-plotted as a scatterplot (Figure 5.10b), a possible first-order response was again observed, particularly for the 5 μM Cd experiments. To verify this hypothesis, Eq. 5.6 was fit to this data using the previously indicated method, with the exception that PC_2 concentration was used in place of C_{Cys}. Results from this fit are shown in Table 5.7, which indicated that the model (Eq. 5.6) explained a large amount of variability in the temporal differences with R^2 values of 0.97 or greater for all of the Cd concentrations. Therefore, the temporal response of xylem sap PC_2 appeared to obey first-order kinetics. Taken together, phytochelatins ($n = 2$) were detected in the xylem sap of *B. napus* under different Cd concentrations and increased over time after initial Cd exposure.

The remaining known thiols that were resolvable using this HPLC method, γ-EC and GSH, were not as consistently detected in xylem sap. In the case of γ-EC, it was only sporadically detected in the xylem sap, and when it was detected, it was at relatively low concentrations and did not appear to display a response to Cd (data not shown). Additionally, GSH, or at least GSH that was in the same form as our

Table 5.7. Results from fitting Eq. 5.6 to the xylem sap PC_2 data

Cd concentration (μM)	C_1 (nM)	k $(h)^{-1}$	R^2
5	75.0	0.0544	0.97
1	19.0	0.0403	0.99
0.5	24.3	0.0307	0.99

analytical standard, was not found in the xylem sap. However, there was clear evidence for an unknown thiol species that eluted off of the HPLC column at nearly the same time as our GSH standard. The differences in elution time between our GSH standard and this unknown thiol species were consistently between 0.2-0.3 min. Thus, this unknown thiol was termed "GSH-like." Approximate concentration of this species was determined by using the GSH calibration curve, and results are shown in Figure 5.11. In the 24 h group, ANOVA found evidence for differences among the treatment means ($p = 0.033$). However, none of the treatments were found to differ from the control, and therefore further comparisons using the Tukey-Kramer procedure were meaningless. At 48 h, the analysis failed to reject H_0 ($p = 0.16$) in favor of H_A; therefore, no statistically significant differences were found among the results in this treatment group. At 72 h, data was log transformed to stabilize data variance, and the *F*-test indicated significant differences among the means ($p < 0.001$). Tukey-Kramer pairwise comparisons indicated significant differences between the control and all 72 h treatments. Further differences were found between the 0.5 μM vs. 5 μM as well as the 1 μM vs. 5 μM Cd treatments. In addition to this "GSH-like" thiol, another unknown thiol-containing species that eluted 0.7 min after cysteine was frequently present in our chromatogram. However, like γ-EC, this thiol was not always detected and approximate concentrations of this thiol always appeared to be about 500 nM or lower (data not shown). Furthermore, the absence of this thiol from certain samples made it difficult to ascertain if there were any patterns in the response to Cd.

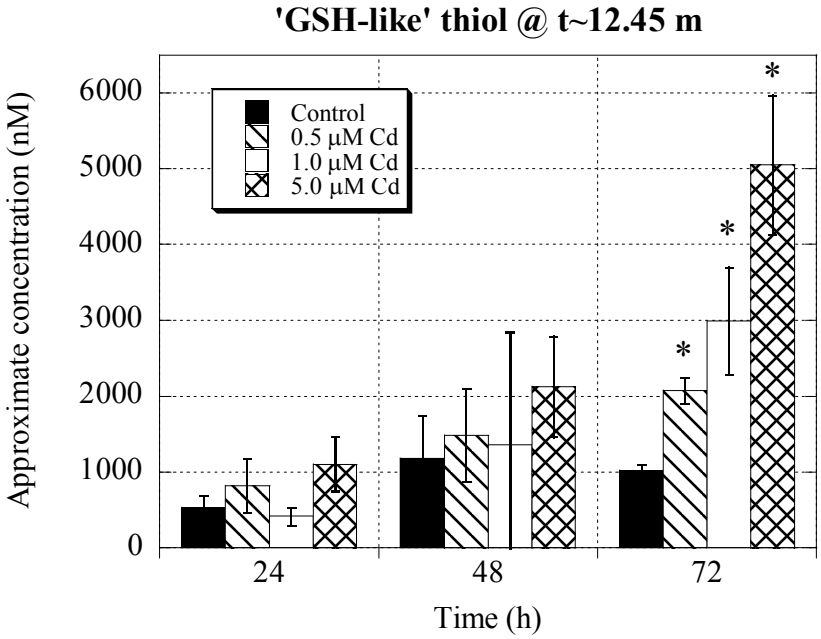

Figure 5.11. Approximate concentration of the "GSH-like" thiol species in the xylem sap of *B. napus*. An asterisk (*) indicates a statistically significant difference from the control within a time period. Error bars represent the standard deviation of the mean.

5.4. Discussion

In this study, we have used a flowing culture system to expose 29-31 d. old *B. napus* to three concentrations of Cd for three different exposure periods, and xylem sap after these Cd exposures was examined for the presence of cysteine, GSH, γ-EC, and PC_2. Additionally, Cd uptake characteristics of *B. napus* plants were monitored using ICP-AES as a way of tracking how much Cd had absorbed while in the hydroponics trough system. For thiol measurements, we used a well established and sensitive HPLC technique that fluorescently labeled thiols with mBrB; our system was capable of thiol measurements down to 5-10 pmole in a 100 μL volume. Cd concentration was also monitored in the trough system during all Cd exposure experiments. Initially, this was done as a way of roughly monitoring how much Cd was entering the plants, which provided evidence that any observed effects in xylem sap were likely due to the Cd that had entered the plants. However, inspection of this depletion data strongly suggested that additional insights into the nature of Cd uptake in *B. napus* were possible. For example, when data such as that presented in Figure 5.6 was analyzed, it was apparent that different rates of depletion, or biphasic kinetics, were occurring in the trough system. In Figure 5.6, a rapid phase of depletion during the first 30 minutes was followed by relatively linear depletion phase through 7 h. While depletion beyond this linear phase was typically more difficult to characterize, the first two phases, characterized by rapid depletion and then a steady decline in concentration were always present in the depletion data.

Plant-free control experiments demonstrated that evaporation as well as Cd interactions with trough components exerted a negligible influence on bulk Cd concentration. Additionally, based on previous work presented in chapter 4, Cd was known to be capable of binding to the root apoplast of *B. napus*. This binding was

shown to occur rapidly, and was modeled using Eq. 4.11 in chapter 4. Therefore, it was possible this binding was responsible for the observed depletion trends, and experiments were conducted using heat-killed roots of *B. napus* to observe the expected magnitude of nonspecific binding within the trough system. Results from these experiments, shown in Figure 5.3, demonstrated that nonspecific binding rapidly occurred in the troughs at all of the tested Cd concentrations. This binding occurred within the first 20-45 minutes as evidenced by the rapid drop in trough effluent Cd concentration that was followed by a period in the trough system whereby Cd concentration stabilized. In particular, time periods between 1-24 h showed significant stability in the trough system, and bound Cd during this time was calculated to be between 10-30% of total system Cd, depending upon concentration. Beyond 24 h, though, bulk Cd levels were less stable, and in most cases, exhibited measurable increases between 24-72 h. However, given that these dead root experiments were conducted over long periods of time (72 h) under normal plant growth conditions in the growth chamber, root degradation and subsequent release of bound Cd is a likely explanation for the observed change in bulk solution concentration. Dead root r_1' values were higher than r_1' values in live roots experiments, which may have indicated that boiling allowed more rapid access to cortical cell and stele cell wall binding sites. However, Cd concentration data suggested that overall levels of binding that resulted from this initial depletion rate were similar between live and dead-root experiments, which indicated that the total number of binding sites was unchanged by boiling. Taken together, data from heat killed roots indicated that, while nonspecific binding was capable of absorbing a certain amount of Cd in the system, it was not capable creating the Cd depletion profile observed in the live-root experiments.

Based on this evidence then, linear depletion that occurred after the period of rapid adsorption was very likely due to uptake into the symplast mediated by membrane transporters. Depletion data were fit using a simple two-parameter model (Eq. 5.2) and uptake results were normalized to root weight and presented in Table 5.3. From this table, it was apparent that Cd uptake was a positive function of concentration; the highest Cd concentrations resulted in the highest uptake rates. The average rates versus initial Cd concentration were plotted in Figure 5.7b, and this relationship did not appear to be linear. Instead, the relationship showed a saturable response, which was illustrated by the nonparametric curve fit calculated by KaleidaGraph. Furthermore, the parameter values that were obtained from fitting the Michaelis-Menten rate expression to the uptake data were within the range of values that have been reported for Cd uptake in the literature (Cataldo et al. 1983; Cohen et al. 1998; Lombi et al. 2002). These results, while not conclusive, supported the hypothesis that the linear period of depletion represented membrane mediated uptake through a saturable transport system. To this author's knowledge, no other work has shown that saturable Cd uptake kinetics can be resolved using depletion measurements in a flowing culture system, although flowing culture systems have been used to resolve uptake kinetics of other ions such as nitrate (Raman et al. 1995b). It is worth noting that the flowing culture experiments presented here were optimized for xylem sap analysis rather than for Cd uptake kinetics; additional Cd concentrations would be needed in order to get better estimates of the parameters K_m and V_{max}.

Nevertheless, valuable insights were obtained from the collected Cd depletion data. First, Cd uptake kinetics were resolvable through depletion measurements since it was possible to separate the apoplastic and symplastic uptake signals. Second, system volume (V) and root mass (m_r) are critical, and controllable, design parameters. Total system volume determines the total number of moles that are

present, and therefore what percentage of moles may be bound by the apoplast. These apoplastic binding considerations are particularly important for divalent cations such as Cd, since Cd binds to ionized carboxylic groups from organic acids in the cell wall (Demarty et al. 1978; Shomer et al. 2003). Furthermore, experiments optimized for uptake could have used less root mass (or a larger volume of solution), which would have resulted in nonspecific binding representing a smaller overall fraction of total system Cd. However, a careful balance must be achieved between root mass and system volume; too much root mass may result in apoplastic binding dominating the depletion profile while too little root mass or too large of a system volume may result in depletion that is not detectable over short time periods (within several hours). Other design parameters, such as flow rate, are important as well; in a recirculating system such as the one used here, the main contributions of flow rate are (1) to ensure that mass transport limitations to the root surface are minimized and (2) to maintain a low enough average retention time in the system so that system solution is always adequately mixed. Furthermore, the ionic background is important; these experiments were conducted using a complete nutrient solution that was only lacking Cu^{2+} and Fe-HEDTA in order remain consistent with previous work as well as eliminate additional Cd-binding ligands within the system. Work conducted in the batch-based system that was described in chapter 4 found that binding was more rapid in solutions that contained only Cd, due to less competition for binding sites. The same phenomenon would likely be observed in the trough system, which would require additional testing and adjustment of design parameters.

Results of our thiol analysis indicated that thiols exist in measurable quantities in *B. napus* xylem sap. This was particularly the case with cysteine, as control plants routinely exhibited concentrations of around 800 nM. Furthermore, xylem sap cysteine concentrations showed a strong response to Cd exposure, which was

particularly evident by 72 h after initial introduction of Cd into the trough system. At earlier time periods, results were less obvious, but an elevated response by 48 h was evident in the 1 μM and 5 μM Cd treatments. The 0.5 μM Cd treatment, however, was not strong enough to elicit an apparent response by 48 h. Furthermore, cysteine concentrations showed no discernable response at any of the tested Cd concentrations at 24 h, which indicated that more than 24 h were required after Cd exposure before cysteine responses became evident in xylem sap. Temporal responses in concentration, at least for the 1 μM and 5 μM Cd treatments, were well described by a first order kinetic model (Eq. 5.6). At 0.5 μM Cd, though, first-order kinetics provided less satisfactory results. This may have indicated that a different cysteine response mechanism was present at this lower Cd concentration, or alternatively, additional time periods may have been needed beyond 72 h at 0.5 μM Cd in order to ascertain if the response would be more adequately described by Eq. 5.6. Model parameter C_1, the predicted concentration at $t = 0$, were between 264 and 438 nM, which was within the range of values observed in untreated control plants.

Our results also indicated the presence of PC_2 in the xylem sap. Unlike cysteine, PC_2 results did show a response by 24 h to all of the tested Cd concentrations. In this case, PC_2 was not found in any of the control plants across all time periods, which agrees with the current understanding of how phytochelatin synthase operates and is activated in non-hyperaccumulating plants (Clemens 2006a). Although some have postulated a role for PCs in metal homeostasis (Rauser 1995), our xylem sap results found no evidence for such a function as PC_2 was not detected in the xylem sap of plants that were not exposed to Cd. Alternatively, it is possible that our detection method was not sensitive enough to detect if PC_2 was involved in metal homeostasis or that homeostatic functions do not involve PC_2 presence in xylem sap. Notably, while all Cd treatments were effective in causing a detectable PC_2 response

in xylem sap, the 72 h, 5 μM Cd treatment resulted in a very sharp increase in PC_2 levels compared to other Cd concentrations at that time point. The physiological significance, if any, of this concentration spike was unknown, although 5 μM Cd did represent a severe challenge to *B. napus* as evidenced by leaf chlorosis in the 5 μM treatments at 72 h, and to a lesser extent, 48 h. PC_2 temporal trends were well described by the first-order kinetic model (Eq. 5.6) at all of the tested Cd concentrations. In this case, the model parameter C_1 predicted PC_2 concentrations between 19 and 75 nM at $t = 0$. These values, although low, may indicate that a first-order model does not accurately describe PC_2 data at $t = 0$ since no PCs were detected in control plants.

Therefore, the PC_2 results presented here support a growing body of evidence that suggests that PCs are capable of long-distance transport in plants. The presence of PC_2 in xylem sap further supports the work presented in the *Arabidopsis* PC transport studies (Gong et al. 2003; Chen et al. 2006) by providing some direct evidence of such transport. These results also further validate that direct measurement of PCs is possible in xylem sap, as reported by the recent work of Wei et al. (2007) on *B. juncea*, a relative of *B. napus*. However, some differences between our work and Wei et al. (2007) should be noted. First, we did not attempt to conduct a metal speciation study as it seemed unlikely that it was possible to avoid significant disturbance to the native xylem sap chemical equilibria using our sampling and detection methods. Furthermore, our detection method relied upon labeling thiols with mBrB followed by separation on a C_{16} column and use of a fluorescence detector, rather than size exclusion chromatography followed by a UV detector. Wei et al. (2007) found evidence for very few PCs in the xylem sap of *B. juncea* that was exposed to 10 μM Cd, and concluded that 1% of the Cd was associated with PCs under this exposure. The work presented here detected PC_2 at Cd exposures down to

0.5 μM. However, our Cd exposure times ranged from 24-72 h prior to collection while a 10 h exposure period was used by Wei et al. (2007). Since our work shows the amount of thiols detected increased with time, it seems possible that the shorter sampling time may have played a role in their results. Also, the highest Cd concentration that was used here was 5 μM, while Wei et al. (2007) used a maximum Cd exposure of 50 μM to induce a Cd-stressed condition that resulted in PC detection. Thus, our method, while not capable of resolving the speciation of Cd, may have been more sensitive for thiols. Despite these differences, however, both studies detected cysteine and PCs in xylem sap. More work remains, though, in trying to determine the exact role of these compounds in long-distance Cd transport, which may be both time and concentration dependent.

Xylem sap analysis also indicated the presence of an unknown thiol compound in chromatograms that showed an increasing response to Cd over time (Figure 5.11). This compound always eluted off of the HPLC column at a time very close to that of the GSH standard, and was thus termed "GSH-like." However, because the difference in elution times was consistently 0.2-0.3 minutes, the unknown compound did not appear to be chemically identical to the GSH standard. Plants are known to contain GSH variants, called homoglutathiones, that have different C-terminal amino acids such as β-Ala (Carnegie 1963) and result from different specificities of glutathione synthetase or possibly post-synthesis modifications of GSH (Skipsey et al. 2005). These GSH variants subsequently give rise to homo-phytochelatins (Grill et al. 1986). While xylem sap PC_2 occasionally deviated slightly from the elution time of the PC_2 standard, the deviation was not consistent across samples and was within the usual variation observed in elution times (< 0.15 min.). If homoglutathione were present, it would have been incorporated in PC_2 and thus caused a more consistent deviation in elution time between the sample and PC_2 standard. However, previously published

work (Grill et al. 1986) suggests that homoglutathione (and PCs derived from h-GSH) have only slightly altered retention times on HPLC systems, and therefore the presence of these compounds in the xylem sap of *B. napus* cannot be ruled out. To definitively answer this question, future work should be performed on a HPLC system coupled to a mass spectrometer. Statistical analysis of this GSH-like compound revealed meaningful differences only in 72 h plants when compared to controls. In this group, pairwise comparison procedures also found significant differences between the 0.5 μM vs. 5 μM and 1 μM vs. 5 μM Cd treatments. The fact that statistically significant differences were found only in the 72 h group was likely due to the higher variability seen in the "GSH-like" data, especially in the 48 h group, which subsequently weakened the power of the ANOVA model to detect meaningful differences in concentration among the various Cd treatments.

Taken together, the work presented here provides direct evidence for the presence of cysteine, PC_2, and other thiol-containing compounds in the xylem sap of *B. napus*. Additionally, xylem sap thiol responses generally took 48-72 h to reach levels that were significantly different from controls. Unfortunately, the precise role of thiols in long distance Cd transport is still unclear, although their presence in the xylem sap supports the hypothesis that, under the right conditions, thiols may play important roles in long distance heavy metal transport. This work also demonstrates that, if appropriate design choices are made, flowing culture systems can be used to differentiate apoplastic from symplastic uptake of Cd, and therefore such systems may have use in examining uptake kinetics of toxic metal ions at the whole plant level. Additional work in optimizing this system for uptake kinetics is needed, though, before better estimates of kinetic uptake parameters can be obtained.

6. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

6.1. Summary

This work was conducted within the larger context of trying to improve process modeling and understanding of plant-Cd interactions which may later translate into advances in phytoremediation system design and modeling. Specifically, the research presented in this thesis was directed towards the following goals: (1) development of a flowing culture measurement system to measure Cd depletion caused by *B. napus*, (2) characterizing apoplastic Cd binding and understanding how it influences uptake measurements made in a flowing culture system, (3) determination of the capabilities of a flowing culture system resolve symplastic uptake, and (4) assaying for the presence of thiol compounds in the xylem sap of *B. napus* in order to understand xylem sap thiol response to Cd exposure and time.

The initial design of ECUDS used a 1.7 L volume and showed that Cd depletion occurred rapidly and reached the detection limit of the ISE within 2 h. Furthermore, biphasic depletion kinetics were present in the depletion profiles, which indicated that multiple mechanisms may have been causing the depletion. In support of this, a trough experiment that used dead roots showed rapid Cd depletion, and therefore understanding these mechanisms was key for uptake to be related to a biological process, and led directly to the second research goal.

The batch based system was developed so that apoplastic Cd binding could be studied in a more rapid manner. Using this system, results showed that bulk Cd was quickly depleted from solution, particularly when the bulk solution initially consisted of only Cd. Less Cd was bound in experiments when the bulk solution contained other ions. Temporal trends in Cd binding were described using an equation derived

from mass action principles which assumed a finite number of binding sites on the root surface and an eventual equilibrium condition. Importantly, results from this system demonstrated the binding reaction reached equilibrium in about 15 min.

The finding that Cd binding could reach equilibrium suggested that depletion systems could potentially measure symplastic uptake if sufficient volume was present. This led to design changes in ECUDS so that it could be used concurrently to monitor Cd depletion and expose plants to Cd for the xylem experiments. These design changes involved adding a second trough, increasing system volume to 10 L, and reducing the number of plants used in a trough to 4. Results using live roots showed two distinct features: an initial rapid phase followed by a longer period of gradual depletion. Rates derived from the gradual depletion phase appeared to obey Michaelis-Menten kinetics, and the estimated kinetic parameters, K_m and V_{max}, were within the range of values that has been observed in the literature. Concurrently, xylem sap thiol contents were analyzed for potential responses to Cd exposure. These investigations found that cysteine and phytochelatin showed responses to time and applied Cd concentration. An unknown thiol, termed "GSH-like" due to an elution time that was very close to the GSH standard, also showed similar response trends. The conclusions and potential future investigations, based on these results, are discussed below.

6.2. Conclusions

6.2.1. Initial design of ECUDS

The initial design of ECUDS followed some previously published research (Raman 1994) regarding design considerations in a flowing culture system that was

used for determination of nitrate uptake in rice through the use of an ISE. In particular, the signal-to-noise ratio (the ratio of voltage changes caused by true Cd depletion to voltage changes caused by sensor drift) was maximized by using small system volumes and a relatively large amount of root mass. However, for a divalent, nonessential heavy metal such as Cd that is unlikely to have dedicated plasma membrane transporters, different parameters must be taken into consideration in the design of a flowing culture measurement system. While the lower uptake rates for a heavy metal such as Cd favor further increasing root mass and lowering system volume to maximize the signal-to-noise ratio, apoplastic binding dominates the depletion profile in such a configuration. Thus, the system volume to root mass ratio must be increased to accommodate nonspecific binding considerations. This has two main consequences. The first is that it favors a depletion profile that takes place over a longer time period, and problematically, reduces the ISE signal-to-noise ratio, which, along with the low K_m for Cd uptake, favors the use of other detection methods such as ICP-AES. The second consequence is that only net symplastic uptake of Cd can be detected, rather than unidirectional influx. If there is a short period of unidirectional influx through a plasma membrane transport system, it will be masked by the apoplastic binding signal.

6.2.2. Utility of flowing culture systems for resolving Cd uptake kinetics

This work demonstrated that flowing culture systems are capable of resolving the rapid apoplastic binding signal from a second, slower depletion signal that was resolved into the characteristic kinetics expected from depletion caused by transporter mediated uptake. Furthermore, this was the first known report of using a flowing culture technique to resolve the kinetics of a saturable transport system with a K_m

value at or below 1 μM. Therefore, flowing culture techniques can be used in cases where ions have significant nonspecific interactions with the cell wall. This work also revealed that, because measurements are made after cell wall binding reaches equilibrium, Cd uptake isotherms derived through flowing culture techniques may not suffer from the linear bias present in Cd uptake isotherms derived through procedures that use desorbing solutions. This linear bias may be due to the inability of desorbing solutions to completely remove Cd bound to the cell wall. In short, flowing culture systems may offer a more direct approach to estimating Cd uptake parameters and the contribution of cell wall binding.

6.2.3. Modeling of Cd binding to the cell wall using mass action principles

The approach used in this research to model the Cd binding reaction was based on simple mass action principles that assumed the binding reaction was reversible and was fundamentally governed by an equilibrium process (Eq. 4.5). This modeling approach resulted in a simplified mathematical treatment of the cell wall that ignores some of the complexities of ion interactions with this structurally complex substrate. Furthermore, other investigators have shown that not all Cd binding to the cell wall is reversible (Cataldo et al. 1983) and thus the model assumptions of total reversibility might be violated. However, for depletion measurement systems, more complicated approaches to describing the cell wall are not needed since the main interest is in understanding the behavior of an ion in the bulk solution in relation to the cell wall. In short, the specific details of an ion exchange reaction, such as the precise identities of which two ions are exchanging, do not necessarily need to be modeled in order to describe overall behavior of the forward binding reaction. The temporal Cd binding model used here accomplished this goal.

6.2.4. Use of heat killed roots to estimate apoplastic Cd binding

The method used in this work exposed plant roots to boiling DI water for 3 min in order to eliminate the contribution of Cd uptake caused by plasma membrane transporters. This potentially had the effect of altering apoplastic binding characteristics, which would have resulted in differences between such binding in live and dead roots. However, experimental data did not reveal significant alterations in binding properties between live and dead roots. In trough experiments, both live and dead roots caused rapid initial depletion of system Cd. The primary difference was in the initially higher r_1' values calculated for the heat-killed roots shown in Table 5.3. This higher rate of initial apoplastic binding is in agreement with results obtained from methanol-chloroform treated pea roots (Cohen et al. 1998). Despite this, total apoplastically bound Cd did not appear to significantly differ between live and dead roots in the trough experiments. Thus, the initially higher binding rate of dead roots indicated more accessible binding sites, while similar amounts of bound Cd suggested that the total number of binding sites were relatively unchanged between live and dead roots. Short treatments in boiling DI water, then, represented an effective way to isolate and study the apoplastic uptake signal in this work.

6.2.5. Cd effects on xylem sap cysteine concentrations

Control plants at all time points contained measurable concentrations of cysteine in the xylem sap. This indicated that cysteine is usually present at basal levels. The reasons behind this presence are unknown; cysteine may have trafficking functions, signaling functions, or be destined for incorporation into a protein or nonprotein thiol molecule such as GSH. Cysteine concentrations showed a time and

dose dependent response to Cd concentration. Whether or not the role of xylem sap cysteine changes in plants that are experiencing Cd stress is also an unresolved question. The cysteine response observed in this study makes it tempting to speculate that cysteine may serve as a potential binding ligand to Cd, although this hypothesis awaits experimental confirmation.

6.2.6. Cd effects on xylem sap phytochelatin concentrations

In *B. napus* plants that were exposed to Cd, PC_2 was detected in the xylem sap. Like cysteine, PC_2 showed both a time and dose-dependent concentration response. No PCs were detected in the xylem sap of control plants that had not been exposed to nutrient solution with added Cd. This result is consistent with current understanding of how phytochelatins are synthesized in response to heavy metal exposure. The presence of PC_2 in the xylem sap was intriguing since the role of phytochelatins in long-distance heavy metal transport is unclear. However, there is a growing body of evidence that suggests phytochelatins are capable of long distance transport in plants, in both root-to-shoot and shoot-to-root directions. There is also evidence they may have a role in heavy metal transport. The direct observations of PC_2 presence in the xylem sap, along with the concentration responses that were observed, indicates an active response and likely role of PC_2 in xylem sap transport of Cd. The potential xylem sap responses of longer chain PCs are, at this point, open questions.

6.2.7. Other thiols

Thiol compounds that could not be identified by the methods used in this work were found in the xylem sap of *B. napus*. One of these thiols consistently eluted from

the HPLC column 0.2-0.3 min after the GSH standard, and was thus termed "GSH-like." Similar to cysteine and PC_2, this thiol showed a time and dose dependent response to Cd, although more noise was present in the data. Because the identity of this compound is unknown, it is impossible to identify or speculate about potential biological roles. Other unknown peaks were occasionally present xylem sap chromatograms, but none exhibited a consistent presence or response to Cd. Use of a HPLC coupled to a mass spectrometer would help in identification of these unknowns.

6.3. Future studies

The flowing culture system used here provides a very good platform for further development in investigations into both plant Cd uptake processes as well as additional xylem sap investigations. Since characteristics of Cd binding to the apoplast are now understood, the dynamics of Cd uptake kinetics can now be explored using this system. For instance, the temporal dynamics of Cd uptake processes—i.e. the time-dependent effects of Cd exposure on uptake parameters—should be investigated to provide useful insights into plant responses to Cd toxicity from acute and chronic exposure perspectives. Potentially, plants bred or genetically engineered to have better uptake properties and tolerance, particularly over long exposure periods, could be tested for such performance in this system, which provides a platform to model and quantify such properties. For instance, plants manipulated to constitutively express high levels of ion uptake transporters could be studied using this system as a way to provide kinetic information to assess the efficacy of such efforts. Furthermore, other parameters that impact Cd uptake such as nutritional status, pretreatment, and environmental factors should be investigated in order to obtain more insight into

whole plant interactions with Cd. Ultimately, better models will result from such efforts.

Additional work could also go into further design and development of the trough system. Computer controlled valves could be reintroduced to facilitate pretreatment strategies or simply eliminate the manual fluid selection that was used. Although solution sampling followed by ICP analysis may be the preferred detection method, an automated sample collection system could be added to the troughs. This would expand the time periods over which measurements could be taken, such as during overnight hours. Different experimental strategies could also be tried for uptake measurements since the time frame for cell wall binding to reach equilibrium is now understood. The system could be engineered to have two configurations: a large volume one for cell wall saturation, followed by a smaller volume configuration, using a solution of identical concentration, for uptake measurements. This type of approach may allow for multiple uptake readings in the course of a single day by either using solutions of identical concentrations, or progressively higher ones. In the latter case, each successively higher concentration would need to be preceded by a large volume solution to saturate the cell wall. Clearly, multiple options exist for such a system.

Finally, additional efforts should be directed towards better understanding the role (or roles) of thiol compounds in the xylem sap of *B. napus* in response to Cd exposure. Again, dynamics and long term temporal trends in xylem sap transport properties and their associated ligands are poorly understood. Xylem sap concentrations of cysteine and PC_2 appeared to follow first order kinetics in concentration after Cd exposure, but how long this response would be maintained is unknown. Furthermore, the importance of thiol compounds under chronic Cd stress is not understood, and the flowing culture system here could be used in such studies. The presence of PC_2 should be particularly investigated for its potential role in long

distance Cd transport. Despite difficulties in developing an experimental method that does not alter xylem sap chemical equilibria upon collection and separation, further speciation studies would provide direct evidence for PC-Cd transport at low Cd concentrations. Xylem sap PCs also raise an important question related to molecular biology: how do these compounds enter the xylem? Presumably, a PC or PC-Cd complex would enter through a transport protein, but a transporter (or possibly multiple transporters) capable of this function have not been identified. Finding a xylem loading transporter for PCs or PC-Cd complexes and understanding its regulatory mechanisms would represent one step towards potentially manipulating shoot Cd accumulation. Resolving such questions will provide good targets towards engineering efforts to develop plants with better metal accumulation properties.

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