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Proteomic Approaches to Elucidate Molecular Mechanisms of Metal Accumulation in Plants with Focus on Arsenic

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**PROTEOMIC APPROACHES TO ELUCIDATE MOLECULAR MECHANISMS OF
METAL ACCUMULATION IN PLANTS WITH FOCUS ON ARSENIC**

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DEDICATION

*This work is dedicated with much love and affection to my beloved parents
and my loving husband.*

ACKNOWLEDGMENT

Many individuals have been supportive and instrumental in assisting me with this work, and I owe them a debt of gratitude. I am deeply thankful to my research supervisor, Dr. Robert Wei, whose guidance, advice, encouragements and his support throughout my PhD career have been truly exceptional. I am deeply indebted to my co-adviser, Dr, Harry van Keulen, for giving me the opportunity to carry on this research in his lab, and also for his help, stimulating suggestions, and encouragement that helped me in all the times of this research and writing of this thesis. It would not be an exaggeration to say that if he had not been there, I may not have reached the finishing line.

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PROTEOMIC APPROACHES TO ELUCIDATE MOLECULAR MECHANISMS OF METAL ACCUMULATION IN PLANTS WITH FOCUS ON ARSENIC

ABSTRACT

Environmental pollution is a continuing global problem. Among various pollutants, toxic heavy metals contribute to a great proportion of soil and water pollution and causes major problems to human beings, animals as well as to plants. It is becoming a critical concern and needs rapid and effective remediation. The conventional remediation methods are no longer economically valid and practical for environmental cleanup, therefore, they have to be replaced with an advanced and modern technology and that is: “using plants”, which is referred to as “phytoremediation”.

This study is focused on arsenic (As) and lead (Pb) pollution and the prospect of remediation by using certain plants that are capable of accumulating extraordinary high amounts of toxic heavy metals. This is referred as to “heavy metal hyperaccumulation”. Unfortunately the biochemical and physiological mechanisms responsible for this phenomenon are still not completely understood. Therefore, investigation on the involvement of the plant’s proteome in metal accumulation, stress, and tolerance offers a new platform to understand the responsible biochemical and physiological mechanisms. This is the foundation of this dissertation.

The Dwarf Sunflower, *Helianthus annuus*, was used to study the proteomic responses of the plants to exposure to As and Pb. Differential expression of several proteins was shown by the use of one- and two-dimensional gel electrophoresis coupled with tandem mass spectrometry. Polyethelene glycol fractionation was used as the most suitable protein extraction method for Sunflowers. The enzyme chitinase was one of the first proteins identified that show a

reproducible up-regulation in response to both As and Pb. This protein is involved in a general defense mechanisms to stress conditions. Other proteins that were over-expressed were thaumatin, heat shock protein, ATP synthase α and β subunits, malate dehydrogenase, carbonic anhydrase, RuBisCO activase, triose phosphate isomerase and nitrophenyl phosphatase. All these have vital roles in a metabolic response to metal stress.

A model organism, *Chlamydomonas reinhardtii*, was chosen to study at the cellular level the molecular mechanism of response to As exposure. Proteins, over-expressed and induced by As stress, were associated with the removal of damaged proteins, oxidative stress, increased energy demand, protein synthesis and protein folding. The majority of the cell's reaction appears mainly to be a response to protein and oxidative damage which is only slightly different from what was observed for Sunflowers.

Finally a known arsenic hyperaccumulator, the Fern *Pteris vittata*, was used to see if it showed a similar response to arsenate. It also was tested if the amount of arsenate makes a difference in the Ferns' response at the protein level. Forty (40) proteins were differentially expressed in response to arsenic exposure and the identified proteins reveal that arsenic predominately affects proteins belonging to photosynthesis, carbon metabolism, protein turnover and some proteins that are generally recognized as stress-related. Thus, even the hyperaccumulating Fern deals with arsenic toxicity by up-regulation of metabolic pathways and general damage repair pathways.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 ENVIRONMENTAL POLLUTION

Environmental pollution is a continuing global problem and can be described as the introduction of harmful substances into the environment accidentally or intentionally and will result in disturbing the stability, order and comfort of the ecosystem. This also has a major impact on human health in addition to that on the entire ecosystem. Water, air and soil are polluted with a broad range of contaminants which can be divided into four classes: Nutrients (Phosphorous, Nitrogen), organic contaminants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls and pesticides), xenobiotics derived from the pharmaceutical industry (personal care products, hormones) and metals and metalloids (Cu, Zn, Fe, Cd, Ni, Pb, Hg, Cr, Sr, Al, Ba, Se, and As) (Marchand et al., 2010).

Elevated levels of contaminants in soil and aquatic ecosystems cause significant concern with respect to plants and aquatic living beings because pollution results in uptake and subsequent entry into wildlife and human food chains. Human beings are the most affected because of

accumulation of the highest concentrations of contaminants by the process of biomagnification (Pergent et al., 1999). This has a serious impact of human health and can result in cardiovascular disease, cancer, respiratory problems, skin problems and a variety of other illnesses. Environmental pollution also has a major effect on agriculture. Soil is being seriously degraded as a result of industrial and urban waste release, civil activities and, in addition, natural disasters such as volcanic eruptions and geochemical weathering of rocks (Vamerali et al., 2010; Ahsen et al., 2009). This leads to a constant soil contamination and degradation which eventually causes the reduction of crop yield, plant growth and their immunity to various diseases as the above mentioned metals can adversely affect metabolic functions including physiological and biochemical processes in plants such as photosynthesis, respiration and cellular maintenance. The contaminants also can diffuse and leech into ground water and ultimately to drinking water sources. Stability of entire ecosystems depends on the equilibrium and well-maintenance of the environmental cycles. Pollution causes a huge damage to the entire world which means its prevention and attenuation is a very important if not timely concern. In this study, the major focus was on toxic heavy metal and metalloid (considered also as metals) pollution in soil and water and the prospect of remediation by using plants as a means to accumulate and concentrate those toxins.

1.2 HEAVY METAL CONTAMINATION IN SOIL AND WATER

Among various organic and inorganic pollutants, toxic heavy metals contribute a great proportion of soil and water pollution all over the world (Vamerali et al., 2010). The term

“heavy” means a series of metals as well as metalloids that can be toxic to both plants and animals even at very low concentrations. The term heavy is a misnomer since all toxic metals fall in this category. Heavy metals are considered as potentially phytotoxic elements (Rascio and Navari-Izzo, 2011). It has been reported that the concentrations of certain metals including cadmium (Cd), chromium (Cr), Nickel (Ni), Mercury (Hg), lead (Pb), copper (Cu), Zinc (Zn), selenium (Se) and arsenic (As) are rapidly increasing as the result of various anthropogenic activities such as vehicular traffic, burning of fossil fuels, soil fertilization, use of pesticides, mining and metallurgical activities, and the disposal of sludge, industrial and agricultural waste (Ahsen et al., 2009; Giordani et al., 2005). Cadmium which enters the environment mainly from industrial processes and fertilizers (Rodríguez-Serrano et al., 2009) has been reported as a toxic trace pollutant for humans, animals and plants. It affects plant growth (Sandalio et al., 2001) and also causes cancer in humans. Aluminum (Al) contributes as the primary factor for land soil acidity (Yang et al., 2007) resulting in substantial reduction of crop yield. Arsenic (As) is one of the principal metalloid toxins in ground water and, thus, drinking water and causes what is now described as a global epidemic of poisoning, causing critical health issues such as skin lesions, cancer and respiratory problems (Alkorta et al., 2004) in many countries throughout the world especially China, Bangladesh, Vietnam, USA and India. Chromium (Cr) is another widely known toxic carcinogen of which Von Burg and Liu have published a toxicological update and how it affects human health in various ways (Von Burg and Liu, 1993). Nriagu and Pacyna (Nriagu and Pacyna, 1998) describe that the annual toxicity of all toxic metals mobilized exceeds the combined total toxicity of radioactive and organic waste produced every year from all sources. This means that metal pollution is of critical concern and needs rapid and effective remediation worldwide.

1.3 CONVENTIONAL METHODS IN METAL REMEDIATION

Most of the remediation processes still make use of conventional physicochemical remediation methods such as excavation, soil capping, burning, reburial, thermal and ion exchange techniques and soil washing. Even though excavation, capping and reburial are described as the most common methods of remediating heavy metal contaminated soil, they are no longer accepted as accurate methods according to the decision reported from the metal-contaminated Superfund sites on the USEPA National Priority List (Neilson et al., 2003). In addition to that, many other drawbacks and limitations are reported such as high cost, inefficiency, destruction of soil structure and fertility (Lone et al., 2008; McGrath et al., 2001). Various surveys and studies around the world have shown that the cost of environmental cleanup using conventional remediation technologies is tremendously high which was \$6-8 billion a year in the US and \$25-50 billion per year worldwide according to D Glass Associates in 1999 (Pilon-Smits and Freeman, 2006). Also the Economic Research Service of the U.S. Department of Agriculture reports that the cost of current hazardous waste site cleanup with conventional technologies varies from \$400 to \$750 billion in the United State only and further mentions that the remediation of heavy metal contaminated sites would cost over \$7 billion over the next five years in the USA (Economic Research Service, USDA, 1996). In addition to all these drawbacks, applying the conventional physico-chemical remediation techniques cause extensive damage to the environment such as rendering the land useless for plant growth and creation of additional polluted lands. Therefore, it is very clear that the conventional remediation methods would be no longer valid and practical for environmental cleanup and have to be replaced with an advanced and modern technology and that is: “using plants”, which is referred to as “Phytoremediation”.

1.4 PHYTOREMEDIATION IN METAL REMEDIATION

Plants require essential and non-essential micronutrients for their growth and development. Heavy metal ions such as copper (Cu^{2+}), zinc (Zn^{2+}), Manganese (Mn^{2+}), iron (Fe^{2+}), Nickel (Ni^{2+}) and cobalt (Co^{2+}) are considered essential micronutrients for plant metabolism as well as cadmium (Cd^{2+}), mercury (Hg^{2+}), silver (Ag^{2+}) and lead (Pb^{2+}), but these belong to the non-essential micronutrients. Regardless of being an essential or non-essential micronutrient, when present in excess, both categories can become extremely toxic to plants and cause symptoms such as necrosis, chlorosis, stunting, leaf discoloration and inhibition of root growth (Williams et al., 2000; Yang et al., 2005). Surprisingly, certain plants are capable of hyperaccumulating toxic heavy metals without showing any toxicity symptoms which suggests a developed resistance and tolerance to heavy metals.

Plants that are “Hyperaccumulators” have the ability to absorb and accumulate extraordinarily high amounts of heavy metals in their aerial parts far in excess of the levels found in the majority of plant species while their growth is not affected (Rascio and Navari-Izzo, 2011) and without showing any phytotoxic symptoms (Rascio, 1997). Hypertolerance is one of the key features, allowing plants to avoid heavy metal poisoning and also supports hyperaccumulation. Figure 1 shows how the hyperaccumulators can be distinguished from “excluders” those who retain and detoxify most of the heavy metals in the root tissues with a minimal translocation to the leaves which cells remain sensitive to phytotoxic effects. Hyperaccumulators behave in a totally opposite way with respect to heavy metal uptake and translocation into aerial organs. Hyperaccumulation mainly depends on a few common traits such as 1) greater capability of heavy metal uptake from soil, 2) faster and effective root to shoot metal translocation and much

greater ability to detoxify and sequester huge amounts of heavy metals in the leaves, see figure 1 (Rascio, 1997).

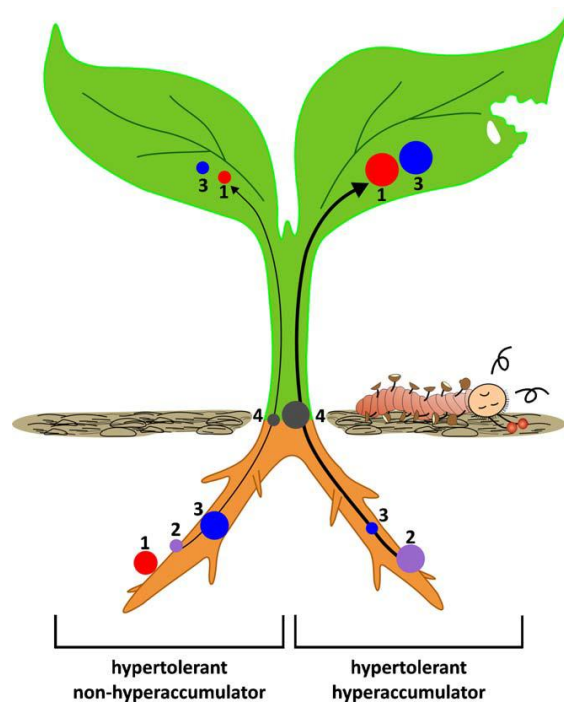


Figure 1: Mechanisms involved in heavy metal hypertolerance and heavy metal distribution in an excluder non-hyperaccumulator and a hyperaccumulator plant.

Mechanisms involved in heavy metal hypertolerance and heavy metal distribution in an excluder non-hyperaccumulator (left) and a hyperaccumulator plant (right) side. (1) Heavy metal binding to the cell walls and/or cell exudates, (2) root uptake, (3) chelation in the cytosol and/or sequestration in vacuoles, (4) root-to-shoot translocation. The spots indicate the plant organ in which the different mechanisms occur and the spot sizes the level of each of them.

Over 450 plant species and over 34 different families have been identified as heavy metal hyperaccumulators of trace metals (Zn, Ni, Mn, Cu, Co and Cd), metalloids (As) and non metals (Se), (Verbruggen et al., 2008) which can be used for environmental cleanup and Table I shows a list of some of the hyperaccumulator plants and the metal/metals they hyperaccumulate from soil and water.

Table I: A list of known metal/metals hyperaccumulators reported from different studies

Plant Species	Metal/Metals hyperaccumulate
<i>Oryza sativa</i>	Al, As, Cd, Cu
<i>Arabidopsis thaliana</i>	Cd, Cs, Cu
<i>Helianthus annuus</i>	Cd, Cr, Ni
<i>Zea mays</i>	As, Cr
<i>Glycine max</i>	Al, Cd
<i>Pteris vittata</i>	As
<i>Thlaspi caerulescens</i>	Zn, Cd
<i>Alyssum murale</i>	Ni
<i>Solanum nigrum</i>	Cd

The use of this inherent ability of plants and their associate microbes to clean up contaminated soil and waters, including ground water, is an emerging *in situ* remediation technology which is referred to as phytoremediation (Economic Research Service, USDA, 1996; Ghosh and Singh, 2005).

The term phytoremediation is derived from the Greek prefix *phyto* (plant), combined to the Latin term *remedium* (to correct or remove an evil) (Ghosh and Singh, 2005). The idea of using plants to cleanup soil and water was first introduced by Chaney in 1983 (Chaney et al., 1997). Here the living plants are considered as solar-driven pumps, which can extract and concentrate heavy metals from the environment (Raskin et al., 1997). In addition, this method is

environmentally friendly, aesthetically pleasing, potentially cheap and offers the possibility of bio-recovery of heavy metals (Yang et al., 2005). There are five main strategies in phytoremediation which are shown in table II including their mechanism, contaminant involved, and medium treated by each strategy (Yang et al., 2005; Ghosh and Singh, 2005).

Table II: List of phytoremediation strategies

No.	Strategy	Mechanism	Contaminant	Medium treated
1	Rhizofiltration	Rhizosphere accumulation	Organics/Inorganics	Surface water & water pumped through roots
2	Phytoextraction	Hyper-accumulation	Inorganics	Soil
3	Phytostabilization	Complexation	Inorganics	Ground water, soil, mine tailings
4	Phytovolatalization	Volatalization by leaves	Organics/Inorganics	Soil, ground water
5	Phytodegradation	Degradation in plant	Organics	Ground water within the rhizosphere and soil

1.4.1 Rhizofiltration

The term “Rhizofiltration” is defined as the use of both terrestrial and aquatic plant roots to absorb, concentrate and precipitate contaminants from polluted effluents in low concentrations (Ghosh and Singh, 2005; Dushenkov et al., 1995). This method is more concerned with the

groundwater remediation rather with that of polluted soil. The main advantage of using this technique is its ability to be used as *in-situ* or *ex-situ* application as well as the opportunity to use species other than hyperaccumulators (Ghosh and Singh, 2005). The *in-situ* method can be applied to surface water rhizofiltration when the plants are grown directly in the contaminated water body and also when ground water is localized within the rhizosphere (root-zone). The pollutants can be adsorbed onto the root surface, absorbed into root tissues or precipitated by the exudates (liquids released from plant tissues) (Ralinda R. Miller, 1996). This technique has been used for Uranium (U) remediation with sunflowers (*Helianthus annuus*) and beans (*Phaseolus vulgaris* L.). Lee and Yang (2010) published that 80% of the initial U in the treated water was efficiently removed by sunflowers within 24 hours and the residual U concentration of the treated water was lower than 30 µg/L which is the limit set by the USEPA for drinking water. For beans, the removal efficiency was roughly 60-80% (Lee and Yang 2010). Also roots of hydroponically grown terrestrial plants such as Indian Mustard (*Brassica juncea* L.) and some grasses can be used to remediate toxic heavy metals such as Cu^{2+} , Cd^{2+} , Ni^{2+} and Pb^{2+} from aqueous solutions (Dushenkov et al., 1995).

1.4.2 Phytoextraction

This is considered as a *green* technology, which was born 15 years ago from the research studies of Raskin et al., (1994) and described as the uptake of pollutants, mainly metals, from the environment and to concentrate them in roots and translocate them into aerial parts which means harvestable plant biomass (Ghnaya et al., 2007; Zhao et al., 2003). This technique has been

widely used for various metal cleanup strategies from the environment and represents one of the largest economically feasible opportunities to use phytoremediation (Raskin et al., 1997).

Lead is one of the most important environmental pollutants and is not very soluble in water, thus plants can extract very little compared to what is present in the soil. But Punamiya et al. (2010) showed that phytoextraction can be used to significantly increase the uptake of Pb by using Vetiver Grass (*Chrysopoganzizanioides* L.) when colonized with the arbuscularmycorrhizal (AM) fungus *Glomusmosseae* (Punamiya et al., 2010). Furthermore, The Phytotech Inc. (now part of Edenspace Corporation; <http://www.edenspace.com>) in the USA used to exercise phytoextraction for lead at a Superfund Site in New Jersey as well as in a residential site in Dorchester, MA, demonstrating that the application of phytoextraction can be successful in the cleanup of lead from the environment (Schwitzgubel et al., 2002). According to Schwitzgubel et al. (2002), the phytoextraction technique can be fully developed only when the mechanisms of metal tolerance, accumulation and translocation are better understood.

1.4.3 Phytostabilization

This approach mainly focuses on sequestering pollutants in soil near the roots not the plant tissues, thus pollutants become less mobile and bio-available because they are stabilized in the substrate or accumulated in the root tissues. Nedjimi et al. (2009) showed that *Atriplex halimus* could be used for the phytostabilization of cadmium-contaminated salt soils (Nedjimi et al., 2009).

1.4.4 Phytovolatalization

In this process, plants uptake water-soluble contaminants and release them into the atmosphere as they transpire the water. Contaminants may be modified into evaporable and usually less toxic compounds along the vascular system and eventually liberated into the air surrounding the plant. The major advantage of this process is that there is no need of harvesting the plants and, therefore, disposal of metals and metalloids from the site (Kotrba et al., 2009). In order to answer the question raised about the safety of using such strategy, Moreno et al. (2005) showed that there would not be any harm for the local population and the regional environment when they applied a preliminary safety assessment study that evaluated phytovolatalization of Mercury and Selenium (Moreno et al., 2005).

1.4.5 Phytodegradation

This can be further classified into rhizodegradation or enhanced rhizosphere biodegradation and phytodegradation. In this process of rhizodegradation organic pollutants in the soil are broken down by the action of microbes associated with the plant rhizosphere and are converted into harmless products (Susarla et al., 2002). These plants have a very good symbiotic relationship with the rhizosphere which shows an enhanced microbial activity. In the case of phytodegradation, plant enzymes and enzyme cofactors are involved in degradation of the harmful contaminants into less harmful or harmless species. These enzymes include dehalogenase, peroxidase and nitroreductase (Susarla et al., 2002). For instance, Dec and Bollag (1994) describe that these plants can degrade compounds that have aromatic rings with the help

of these enzymes, for example phenols in horseradish and potato can be degraded by peroxidases (Dec and Bollag, 1994).

Though phytoremediation-based strategies are considered as novel tools to use in environmental cleanup, certain drawbacks and limitations have been raised which, leads the scientific community to put more efforts in developing and improving the newest strategies and modifications in phytoremediation. The following table (Table III) summarizes the advantages and disadvantages in phytoremediation (Economic Research Service, USDA, 1996).

Table III: Advantages and Disadvantages of phytoremediation

No	Advantages	Disadvantages
1	Amendable to a variety of organic and inorganic compounds	Restricted to sites with shallow contamination within root zone of remediative plants
2	<i>In Situ/Ex Situ</i> Application possible with effluent/soil substrate respectively	May take up to several years to remediate a contaminated site
3	<i>In Situ</i> applications decrease the amount of soil disturbance compared to conventional methods	Restricted to sites with low contaminant concentrations
4	Reduces the amount of waste to be land-filled (up to 95%), can be further utilized as bio-ore of heavy metals	Harvested plant biomass from phytoextraction may be classified as a hazardous waste hence disposal should be proper.
5	<i>In Situ</i> applications decrease spread of contaminant via air and water	Climatic conditions are a limiting factor
6	Does not require expensive equipment or highly specialized personnel	Introduction of nonnative species may affect biodiversity
7	In large scale applications the potential energy stored can be utilized to generate thermal energy	Consumption/utilization of contaminated plant biomass is a cause of concern

1.5 ARSENIC

Arsenic (As) is a metalloid found in soil and air and as a result contaminates drinking water via ground water and food plants such as rice and is, therefore, a serious health threat. As is highly toxic to all living forms and is present in either inorganic or organic forms in different oxidation states. Inorganic forms are the most abundant and are mainly trivalent meta-arsenite (As (III)) and pentavalent arsenate (As (V)); organic forms include methylated arsenic acids such as Monomethylarsenic acid (MMA) and Dimethylarsenic acid (DMA) (Flora, S.J.S., 2011; Devizcaya-Ruiz et al., 2009). The chemical structures of the four major arsenic compounds are shown in figure 2.

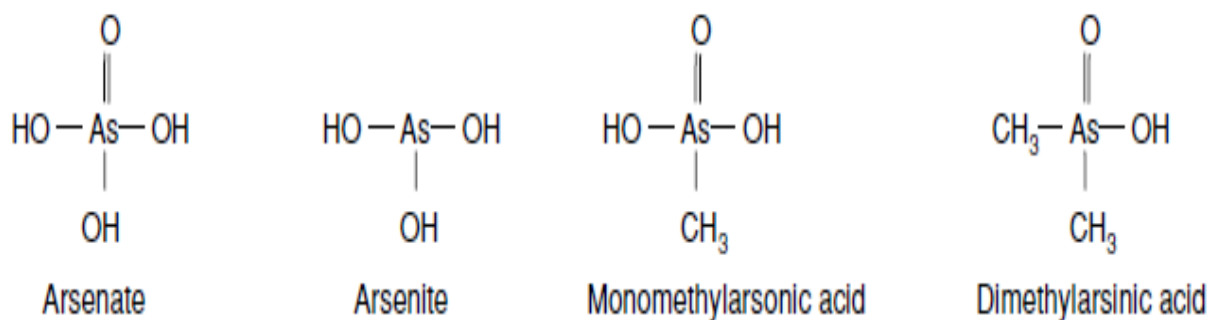


Figure 2: The chemical structures of the most abundant Arsenic compounds

Arsenate and arsenite are the main inorganic arsenic species having oxidation state of +5 and +3 respectively. Monomethyl arsenic acid (MMA) and Dimethylarsenic acid (DMA) are the two major organic arsenic species.

Arsenic contamination of the environment is the result of mining and processing of metal ores (Meharg and Hartely-Whitaker, 2002). In addition, domestic and agricultural lands are highly contaminated with As through the use of pesticides and herbicides which contain arsine (AsH_3) as the major component. Wood preservation, tannery waste release, industrial waste release also add arsenic into the environment in addition to natural sources such as geochemical weathering of rocks and volcanic eruptions. Thus natural and man-made activities contribute each to this worldwide arsenic contamination (Flora, S.J.S., 2011; Hughes M.F., 2002) which has been called a global arsenic epidemic (Alkorta et al., 2004). This, millions of people around the world are being exposed to arsenic every day and the victims are mainly reported from South-East Asia (Bangladesh, Vietnam and West Bengal) where drinking water is primarily coming from ground water contaminated with arsenic (Meharg and Hartely-Whitaker, 2002). This crisis is also reported from many other countries such as China, USA, Mexico, Canada, Hungary, Japan, Argentina, Chile and Taiwan (Jain and Ali, 2000). When As is ingested, soluble As compounds are readily absorbed into the human body and distributed to vital organs such as liver, kidney, lungs, skin and aorta which causes various health issues in respiratory, gastrointestinal, cardio-vascular, nervous and hematopoietic systems. Toxicity symptoms are loss of appetite, jaundice, nausea, diarrhea, dry throat, nervous weakness, erythema and various skin complications like darkened skin exfoliation, falling hair, brittle loose nails, eczema and a horny condition of palms and soles (Jain and Ali, 2000). Moreover, As is a carcinogen which may cause cancer in lungs, skin, bladder, liver and renal organs (Bissen and Frimmel, 2003). Plants also are not resistant to high concentrations of arsenic and various toxicity symptoms such as necrosis, chlorosis, stunting, leaf discoloration and inhibition of root growth are possible.

1.6 ARSENIC UPTAKE AND DEFENSE MECHANISMS IN PLANTS

Little is known about the mechanisms for tolerance of arsenic in arsenic-hyperaccumulator plants and about the mechanisms of its phytotoxicity in non-accumulators. Since arsenate is an analog of phosphate, its toxic effects appear to be largely due to its ability to disrupt phosphate metabolism. Studies with *Pteris vittata* indeed show the potential disruption of arsenic on phosphate metabolism in the fern (Wang et al., 2002). Arsenate, for instance, is taken up via the phosphate transport system (Wang et al., 2002; Poynton et al., 2004), so that the arsenate uptake would decrease with increasing level of phosphate. Further, the higher affinity of phosphate transporters towards phosphate further reduces the uptake of arsenic (Ze-chun et al., 2007). On the other hand, the effect on the phosphate transport system by arsenate would be much less than what may be expected from a simple competition even if the transport system is highly selective for phosphate. In addition, the transport-related phenomenon does not explain the fern's ability to hyperaccumulate arsenic. Arsenate can interfere with various reactions requiring inorganic phosphate as substrate, e.g., ATP synthesis, by forming unstable products with adenosine diphosphate, but *P. vittata* may have special mechanisms to minimize the disruptive effects of arsenic. Another adoptive mechanism against arsenic toxicity appears to be methylation of arsenate, reduction of arsenate to arsenite (Meharg and Hartely-Whitaker, 2002) and induction of thiol-containing molecules such as glutathione and phytochelatins (Zhang et al., 2004). Although arsenate can be reduced to arsenite by a number of reactions such as by glutathione reductase and the recently characterized arsenate reductase (Ellis et al., 2006), arsenite itself can exert toxicity by reacting with sulfhydryl groups leading to inhibition of cellular functions and cell death (Smith et al., 2010), however, this does

not appear to be the case in *P. vittata*. An increased synthesis of sulfur rich small peptides such as phytochelatins (PCs) have been considered not only an important detoxification response to heavy metal exposure in various plants but also for detoxification of arsenic by preventing its uptake, but this mechanism does not seem to account for the ability of ferns to hyperaccumulate arsenic. Research with *Arabidopsis* has indicated that other mechanisms and proteins are likely to be involved with arsenate accumulation, transport and sequestration that would lead to elevated tolerance. One of the most interesting findings of work with *P. vittata* is that arsenite is rapidly removed from tissue by an arsenite transporter which pumps the arsenite into vacuoles (Indriolo et al., 2010). Its evolutionary advantage is probably protection against herbivores.

1.7 PROTEOMIC APPROACHES

Since heavy metal hyperaccumulation by plants has received much interest around the world, various approaches have been taken to study the molecular and biochemical mechanism. During the last decade, transcriptome analysis was one of the main tools used by several research groups to understand the gene expression patterns in plants under heavy metal stress (Sudo et al., 2008; Weber et al., 2006; Abercrombie et al., 2008). But certain limitations may apply in using this method such as poor correlation between changes in the expression of mRNAs and their corresponding proteins (Gygi et al., 1999; Bogeat et al., 2007) due to regulation of protein expression at the translational and or post-translational levels and not simply at the transcriptional level. Since proteins, unlike transcripts, are direct effectors of plant response (Kosova et al., 2011), widening the studies to translational and post-translational levels will offer

a more detailed understanding at the proteome level and will address functional interactions among mature proteins, something that cannot be obtained at the genome level only (Ahsen et al., 2009). This is proven by the fact that proteins not only are involved in enzyme-based changes in metabolite levels, but that they also include components of the transcription and translation machinery as well. Thus proteins regulate plant stress response at both the transcript and protein levels (Kosova et al., 2011).

Investigation on the involvement of the proteome in metal accumulation, stress and tolerance offers a new platform to understand the responsible biochemical and physiological mechanisms due to the fact that changes in protein accumulation under stress conditions are directly interconnected to plant phenotypic responses. This also could be used to introduce protein biomarkers that might indicate quantitative changes in some physiological parameters as the result of stress or toxicity (Kosova et al., 2011). Such markers can be used to compare the proteomes under various conditions of stress such as those resulting from the exposure to different heavy metals, combinations of heavy metals and various concentration ranges of heavy metals. Ultimately this could result in introduction or modification of critical genes in the appropriate plants in order to improve their hyperaccumulation ability as well as other required traits such as fast growing, high biomass, deep roots, easy to harvest, ability to tolerate and accumulate a wide range of heavy metals in their aerial and harvestable parts for effective removal of toxins (Clemens et al., 2002).

According to the review by Jorrín et al., (2007) only one article has been published on plant proteomic response to heavy metals out of seventeen publications on plant proteomics response to other abiotic stress which was a matter of concern to the authors (Jorrín et al., 2007). This might be due to certain technical constraints on proteomic studies such as limitations

inherent to reproducibility in the major tool of proteomic studies which is two dimensional gel electrophoresis coupled with tandem mass spectrometry, heterogeneity of the molecules studied in terms of abundance range, physicochemical properties (extreme values in iso-electric points, molecular mass, etc.) and the unavailability of a fully sequenced genome of the organism (Ahsen et al., 2009). Lately it has been noticed that use of proteomics is slowly gaining confidence within the plant science community by showing an increased growth in the number of publications of proteomic studies on plants in response to heavy metal stress.

Proteins play a major role in cells and contribute to almost all the vital physiological and biochemical functions within the cells. Unfortunately there are many questions that remain unanswered because very little is known about the physiological and biochemical mechanisms of metal accumulation and tolerance.

Therefore studying the proteomic changes as the result of metal-induced stress in plants can reveal how plants are tolerant to toxic heavy metals and what the responsible biochemical mechanisms underlying the heavy metal accumulation are. Several proteins differentially expressed in response to metal stress have been investigated and they appear to be involved in various biochemical pathways. Table IV summarizes which proteins are implicated in response to exposure to heavy metals (Ahsen et al., 2009). This also is discussed in the review on “plant proteome changes under abiotic stress” by Kosova et al. (2011) which summarizes the main results obtained at the proteomic level.

Table IV: Proteins differentially expressed in response to metal toxicity as defined by metal and proteomic analyses

Protein Names	Metals
Proteins involved in antioxidative defense	
APXs, CS, CATs, DHAR, glyoxalase I, GSH1, GPX, GSTs, MDHAR, PODs, Prx, SODs, Trx	Al, As, B, Cd, Cs, Cr, Cu, Mn, Ni
Chaperones	
Chaperonin 60, HSP 70, Dnaj-like proteins, sHSPs, Chaperonin 21, DnaK-type molecular chaperone, HSP 101, PDI	Al, As, B, Cd, Cu, Ni
Proteins involved in signaling molecules and secondary metabolites	
SAMS, ACC oxidase, OPR, CHS, lipoxygenase, ABP19, jasmonate inducible protein-like, auxin induced protein	Al, As, B, Cd, Cs, Cr, Cu
Proteins involved in CO₂ assimilation and photosynthesis	
RuBisCO LSUs and SSUs, RuBisCOactivase	B, Cd, Co, Cu, Hg, Mn

1.8 WHAT IS KNOWN SO FAR?

Several efforts have been made to discover the biochemical and physiological mechanisms responsible for metal accumulation by plants, and its resulting stress and/or tolerance by using proteomic approaches. Some of the major findings will be described here. For instance Ahsen et al. (2009) describe, based on several studies, a number of proposed

defense mechanisms triggered by heavy metal ions and identified proteins corresponding to each mechanism. The suggested mechanisms are shown in Figure 3.

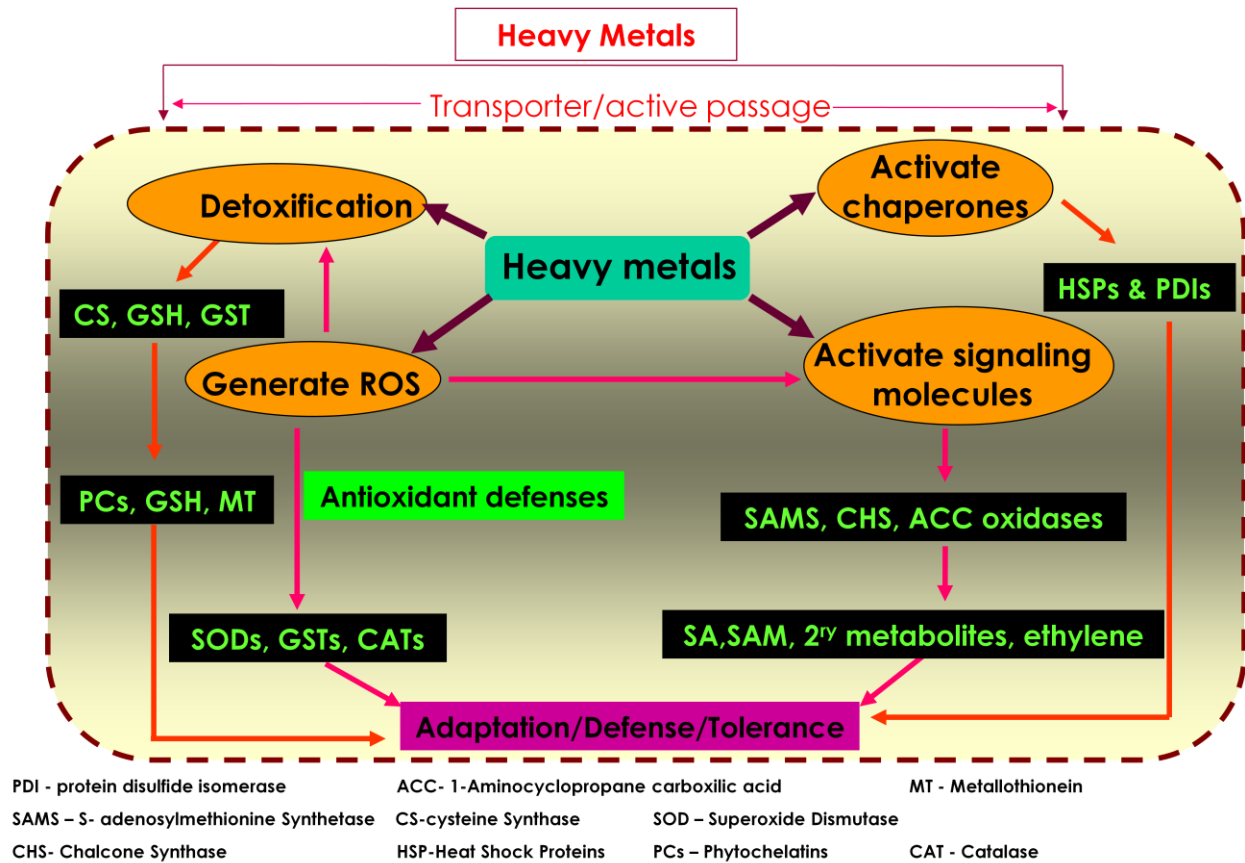


Figure 3: General scheme showing defense mechanisms triggered by heavy-metal ions in plant cells.

After the entry of heavy metals into the cell through active/passive transport and/or by the actions of several transporters, heavy-metal ions generate ROS and activate detoxification mechanisms, signaling molecules, and chaperones, which could eventually lead to an enhanced level of tolerance to the stress

It appears from many studies that heavy metals induce oxidative stress in a number of different ways: 1) transfer of electrons directly in single-electron reactions generating free radicals and reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl free radicals (OH^{\bullet}), hydrogen peroxide (H_2O_2). These are known as, Haber-Weiss or Fenton like reactions which is shown in Figure 4.

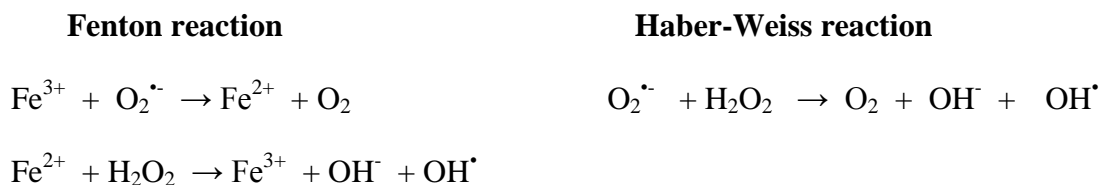


Figure 4: Fenton reaction and Haber-Weiss reaction

Haber Weiss reaction showing the generation of hydroxyl free radicals OH^{\bullet} from Hydrogen peroxide H_2O_2 and superoxide $O_2^{\bullet-}$ and Fenton reaction shows the hydroxyl free radical formation from iron ions.

2) Disturbance of metabolic pathways which also results in increased levels of free radicals and ROS,

3) Inactivation of the antioxidant enzymes (peroxidases (POX), catalases (CAT) and superoxide dismutases (SOD)) responsible for free radical detoxification and

4) Depletion of the non-enzymatic antioxidant Glutathione (GSH) (Bhaduri and Fulekar, 2012).

The ROS are comparatively more reactive than O_2 in that they initiate free radical chain reactions that can damage cells through unspecific oxidation of various biomolecules such as proteins, membrane lipids and DNA. The oxidative injury results in loss of cellular homeostasis and has an effect on plant growth and development.

Activation of the anti-oxidative defense systems is one of the proposed and proven mechanisms. It is composed of different non-enzymatic metabolites such as ascorbate, glutathione (GSH) and tocopherol as well as enzymatic scavengers like SOD, POX and CAT (Bhaduri and Fulekar, 2012; Schützendubel and Polle, 2002). These enzymes and antioxidants are capable of terminating the free radical chain reactions by removing free radical intermediates and so inhibit other oxidation reactions of CAT (Bhaduri and Fulekar, 2012). SODs and ascorbate peroxidases (APXs) act as a first line of defense against ROS in which SOD convert $O_2^{\cdot -}$ into H_2O_2 and APX reduces H_2O_2 to water using ascorbate as a specific electron donor (Asada, 1992). The up-regulation of SOD and/or APXs has been found in several proteomic studies in which various plant species were exposed to a number of different heavy metal including Al in rice (Yang et al., 2007; Fukuda et al., 2007), As in maize (Requejo and Tena, 2005), Cd in poplar (Kieffer et al., 2008) and Cs in *Arabidopsis* (Le Lay et al., 2006). In my study of *Chlamydomonas reinhardtii*, which was used as a model organism to study the effect of arsenic stress, SOD was one of the key proteins that was up-regulated upon arsenic exposure, especially at high concentrations of As.

Degradation of H_2O_2 is done by CAT, which is present in peroxisomes and mitochondria. Therefore, the balance between the activities of SOD, CAT and different peroxidases together with methods of metal sequestration is very important in preventing the generation of hydroxyl free radicals and resulting damage (Mittler, 2002). These enzymes work in concert with glutathione reductase and regenerate ascorbate and glutathione (GSH) (Foyor and Noctor, 2000). In fact, GSH can directly reduce most active oxygen species and chelate metal ions in cells and so protect the macromolecules from damage by free radicals (Freedman et al., 1989).

Heavy metal chelation by metal binding proteins and peptides is another emerging mechanism for heavy metal homeostasis and detoxification in plants (Fig 5) (Yang and Chu, 2011). Metallothionein (MT) and Phytochelatin (PC) are the two best characterized metal binding peptides identified and are low molecular weight (4-8 kDa) peptides with a high content of cysteine (cys). The thiol groups are critical to binding metal ions. When heavy metals like Cd^{2+} and some other heavy metals such as As, Hg and Ag (Cobbett, 2000) enter into the cytosol, the synthesis of PCs from Glutathione by phytochelatin synthase (PCS) is stimulated (Yang and Chu, 2011). This was proven in a study with *Arabidopsis* that GSH-deficient mutants of *Arabidopsis* were PC deficient and Cd sensitive (Cobbett et al., 1998). The metal-PC complexes are sequestered into the vacuole and so the toxicity level is reduced.

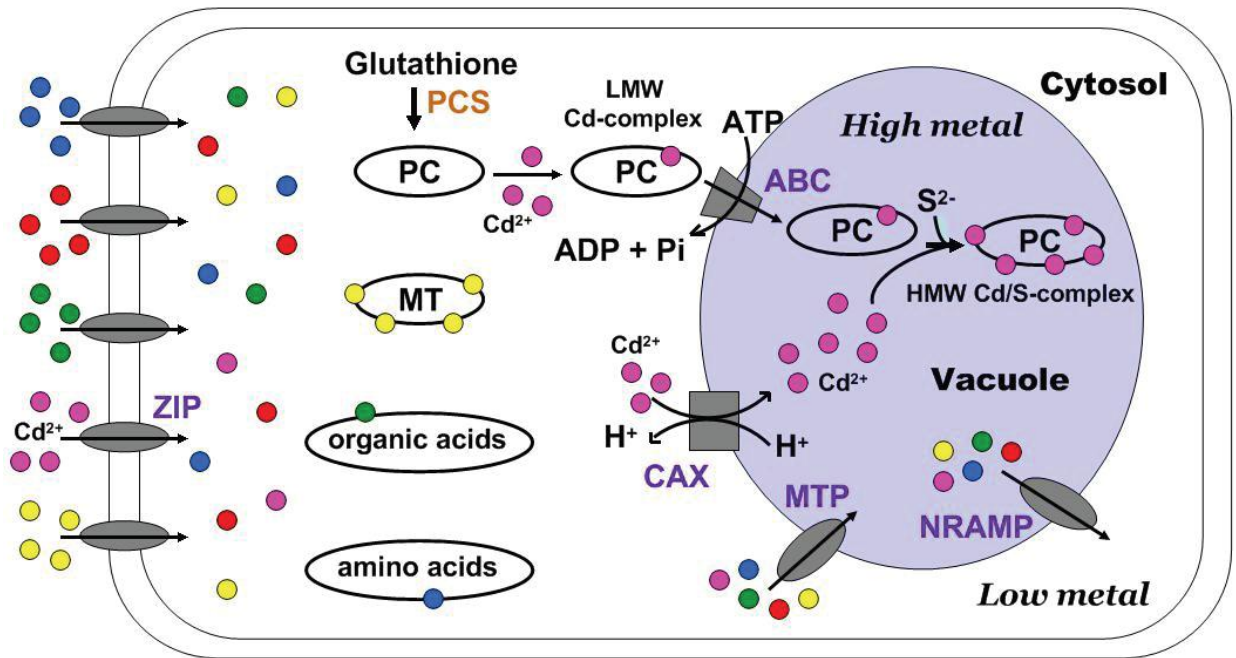


Figure 5: Vacuolar sequestration of heavy metals in plant cells.

Heavy metal ions like Cd^{2+} enter the cytosol and stimulate the glutathione-derived synthesis of phytochelatins (PCs) by PC synthases (PCS). PCs bind cytosolic Cd^{2+} to form the PC-Cd complex which is transported into the vacuole.

Unlike PCs, MTs are cysteine-rich polypeptides encoded by a family of genes and are thought to be involved in metal homeostasis and detoxification. MT gene expression can be induced by certain metal ions such as Cd, Zn, Cu, Hg and Ag and the formed MT can bind to them via metal-thiolate bonds. Among different types of MT, plant MTs belong to type 2 MTs and are characterized by having 2 Cys-rich domains at both N- terminal and C- terminal ends and a large spacer region in between (Peroza and Freisinger, 2007). Though the role of MTs is not established completely, it definitely plays a role in metal detoxification and may have distinct functions for different metals. In addition to forming Metal-MT complexes to reduce the metal toxicity in plants, they could function as antioxidants by direct scavenging of ROS (Yang and Chu, 2011) while playing a role in plasma membrane repairing. Both MT and PC metal complexes are eventually transported into the vacuole which is generally considered to be the main storage site for metals in plant cells and vacuole compartmentalization of metals is also another part of tolerance mechanisms of some metal hyperaccumulators (Tong et al., 2004). The evidence was shown by Krämer et al. (2000) that the Ni hyperaccumulator *Thlaspi goesingense* enhances its Ni tolerance by compartmentalizing most of the intracellular leaf Ni into the vacuole (Krämer et al., 2000).

The plant photosynthetic apparatus and the energy pathways also are severely affected due to the heavy metal accumulation as observed in reduction of chlorophyll content and effects on the ultra-structure of chloroplasts, resulting in a lowering of photosynthetic capacity (Kieffer et al., 2008; Bona et al., 2007). This is shown by degradation of RuBisCO (van Keulen et al., 2008) and decrease in its large and small subunit, up-regulation of the proteins involved in regeneration of ribulose 1,5 biphosphate such as 3-epimerase and ribulose-5-phosphate

isomerase, down regulation of proteins involved in carbon metabolism and photosynthesis such as the oxygen evolving enhancer proteins and aldolase (Keiffer et al., 2008).

Protein structural and functional changes such as protein misfolding, unfolding and malfunction are highly possible in plant cells in response to metal exposure due to the strong reducing effect of some of the heavy metals by which they can cause cleavage of disulfide bridges, interaction with hydroxyl and carboxyl groups of the protein (Sahr et al., 2005). Molecular chaperones, heat shock proteins (HSP) and protein disulfide isomerase (PDI) are synthesized in higher amounts and activated. These are responsible for protein folding, refolding, assembly, reassembly, degradation and translocation, thus involved in the process of protein repair that operates under normal conditions but might be increasing under adverse conditions (Wang et al., 2004). Several groups of HSPs and PDIs are reported as differentially expressed under various heavy metal stress conditions such as Heat Shock cognate 70-1 in *Helianthus annuus* upon Pb exposure (Walliwagedara et al., 2010b), DnaK-type molecular chaperone in response to Cs in *Arabidopsis* (Sahr et al., 2005), increased activity in HSP70 and chaperonin 60-family in Poplar plants when exposed to Cd (Keiffer et al., 2008). PDIs also play an major role in the maturation of newly synthesized proteins by repairing improperly formed disulfide bonds (Song and Wang, 1995) and are reported to be up-regulated (Ahsan et al., 2007; Ahsan et al., 2008; Sarry et al., 2006)

1.9 OVERVIEW OF THE RESEARCH PROJECT OF THIS DISSERTATION

The long-term overall goal of this project was to elucidate the biochemical mechanisms for toxic heavy metal accumulation and tolerance in plants. During this project the emphasis has started to shift towards arsenic as a toxin for the reasons outlined above. Though efforts have been taken by several research groups, only very little has been discovered so far. This study was initiated by using the Dwarf Sunflower *Helianthus annuus* (the cultivars Teddy Bear and Sundance) because it is a known hyperaccumulator of Cd, Ni and Cr (January et al., 2008). For the proteomics work, hydroponically grown plants were treated with different heavy metals, namely Pb, As, Cd, Cr and Ni alone and in combinations and protein isolated from the plants were analyzed using one- and two-dimensional gel electrophoresis (2-DE). Proteins that appeared to be up-regulated after the plants were exposed to the indicated metals were further analyzed by mass spectrometry. Initial protein separation was done by one-dimensional polyacrylamide gel electrophoresis, but the resolution was limited to identification of proteins that were easy to see on the gels because of their over-abundance, such as chitinase (see below). Protein identification using 2-DE was, therefore, very critical due to many reasons as described in Chapter 2. Because of the sensitivity of 2-DE to contaminating substances it was essential to develop the most appropriate sample preparation method. The key issue was to obtain a high resolution of proteins and reproducible 2-D gels.

Chapter 2 deals with the establishment of the methodology to achieve 2-D gels with a high degree of resolution and reproducibility. After the methodology was established, the method was tested by investigating whether proteins could be identified that were up-regulated in Sunflowers exposed to Pb and As. Proteins were analyzed by 2-D gels followed by liquid

chromatography tandem mass spectrometry and up-regulation of some of the proteins was verified by Western blot analysis and by semi-quantitative reverse transcription polymerase chain reaction. The initial analysis showed the up-regulation of some proteins which are normally activated as the result of biotic stress. Since the Sunflower is a complex organism with different tissues, it was decided to use a single cell organism to identify response and the cellular level. For that reason, a fresh water unicellular green alga *Chlamydomonas reinhardtii* was chosen for this study and the main focus was on the effect of arsenic. The proteomic studies revealed a number of proteins that were up-regulated in response to arsenic (Chapter 5)

The hypothesis is that these results could also be applied to higher plants. In the next chapters it will be shown that arsenic has a distinct effect on higher plants such as Sunflowers, that some of these effects are at the organismal and some at the cellular level. However, both Sunflower and *Chlamydomonas* are not hyperaccumulators of As, actually *Chlamydomonas* is very sensitive to As poisoning, as will be shown in Chapter 5. Therefore, in the final chapter the effect of As will be investigated on a plant that is a known hyperaccumulator of As to see if the same type of stress response is taking place in this plant or that the hyperaccumulator status has a totally different effect of protein synthesis.

1.10 CHAPTER 1 - APPENDIX

The organisms

1. *Helianthus annuus* The Dwarf Sunflower

Cultivars: Sundance Kit and Teddy Bear

Systematics:

Viridiplantae

Streptophyta;

Embryophyta;

Tracheophyta;

Spermatophyta;

Magnoliophyta;

eudicotyledons;

core eudicotyledons;

asterids;

campanulids;

Asterales;

Asteraceae;

Asteroideae;

Heliantheae alliance;

Heliantheae;

Helianthus.

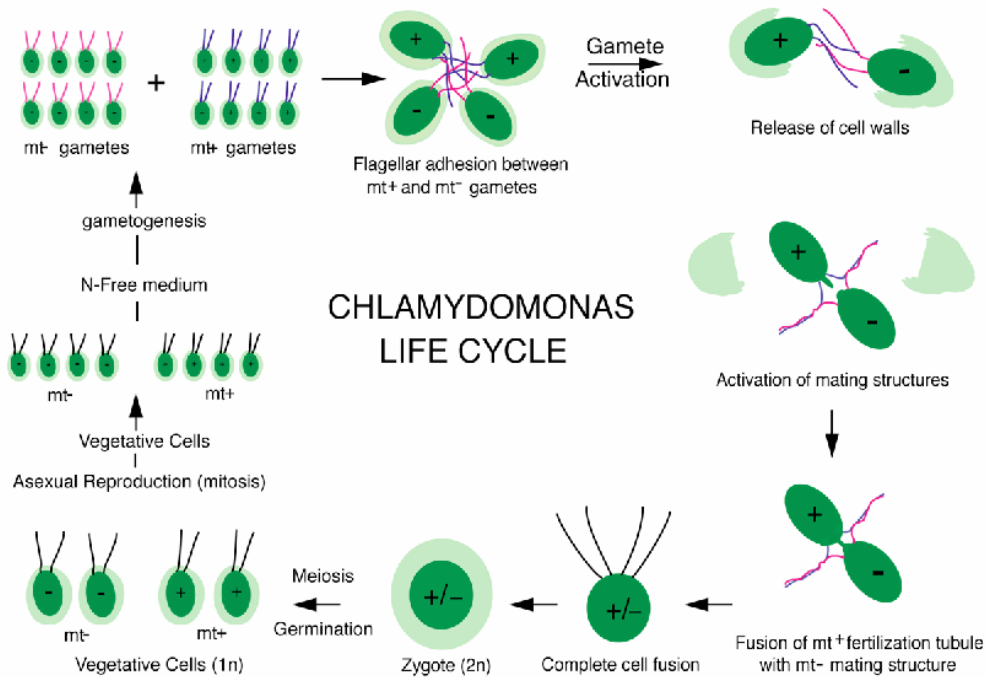


Teddy Bear grown at Cleveland State University

2. *Chlamydomonas reinhardtii* Fresh water Green alga

Systematics:

Viridiplantae;
 Chlorophyta;
 Chlorophyceae;
 Chlamydomonadales;
 Chlamydomonadaceae;
Chlamydomonas.



Chlamydomonas reinhardtii unicellular green alga

3. *Pteris vittata* Chinese Ladder Brake

Chinese Ladder Brake, is native to China. It is found in the Southeastern United States, it is a tropical fern and is an invasive species in Florida, Louisiana, Texas and Georgia. Brake fern is also found in California. The plant is not on the Federal or State Noxious Weed List, but it is listed on the Florida Exotic Pest Plant Council's Invasive Plant List, since 1999

Systematics:

Viridiplantae;
Streptophyta;
Embryophyta;
Tracheophyta;
Moniliformopses;
Polypodiopsida;
Polypodiales;
Pteridaceae;
Pteris.



***Pteris* in Green house at Cleveland State University**



Fronnd with spores



“Fiddle Head”



Gametophytes, grown from spores (May 2012)

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CHAPTER 2

COMPARISON OF SAMPLE PREPARATION METHODS FOR THE RESOLUTION OF METAL-REGULATED PROTEINS IN *HELIANTHUS ANNUUS* BY 2-DIMENSIONAL GEL ELECTROPHORESIS

2.1 INTRODUCTION

The Dwarf Sunflower, *Helianthus annuus*, is a hyperaccumulator of the heavy metals cadmium, nickel, and chromium. The molecular mechanism of hyperaccumulation and adapting to the resulting abiotic stress is largely unknown. Metal-binding and other proteins induced in response to stress conditions may play indispensable roles in allowing the metals to accumulate and the plant to adapt itself.

Various proteins that are responding to the plant's exposure to heavy metals could be revealed by a proteomics approach, using two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry. In this manner gene expression under different physiological conditions, such as environmental stress can be studied. Previously unknown proteins could be involved in metal binding or in general to the plant's response to toxins. Only identification of proteins

expressed under defined experimental conditions could provide insight in stress response. These proteomics techniques have become the principal means to resolve and characterize proteins in such complex matrices as cellular extracts and serum (Person et al., 2006). While the analysis of proteins derived from animal and bacterial sources (Encarnacion et al., 2005) has been the main focus, they have also been applied to a number of plant species including rice (Hajduch et al., 2001) and banana (Carpentier et al., 2005). Compared to microorganisms and animal cells, however, the extraction and resolution of proteins from plant tissues presents a number of challenges due to a comparatively low concentration of protein per weight of tissue (Jacobs et al., 2001), high protease activities (Lopez et al., 2007; Gegenhemier et al., 1990), and the presence of various interfering substances, such as polyphenols and carbohydrates (Carpentier et al., 2005; Jacobs et al., 2001; Michaud et al., 1995; Giavalisco et al., 2003). Furthermore, it appears that the protein composition of each plant species is unique. Therefore, a well-defined set of conditions for each plant species is essential in obtaining reproducible and accurate results. In the 1980s, much effort has been directed towards the establishment of sample preparation methods for plant tissues (Meyer et al., 1988). Sample preparations generally use a one-step protocol, i.e., denaturing extraction in lysis buffer, which was shown to be suitable to obtain clean samples (Carpentier et al., 2005). The majority of the protocols for sample preparation from plant tissues now involve two or more steps. These are precipitation-based (Giavalisco et al., 2003) by the addition of high salt concentrations of divalent ions (Carpentier et al., 2005) followed by treatment of water miscible organic solvents such as acetone and acids (Michaud et al., 1995), but a loss of proteins due to either incomplete precipitation or resolubilization of precipitants could present a serious problem. In addition, the electrophoretic separation of proteins from plant extracts is often complicated due to the presence of many non-protein

contaminants indigenous to the plant, such as organic acids, lipids, polyphenols, pigments, and terpenes (Wang et al., 2003). Moreover, a high abundance of certain proteins, e.g., the large subunit of ribulose1,5 bisphosphate carboxylase/oxygenase (RuBisCO) in leaves, hampers the detection of many and, therefore, also differentially expressed proteins, especially overshadow the identification of less abundant, proteins in 2-DE (Vincent et al., 2006).

The main purpose of this study was to test and optimize sample preparation methods suitable for 2-DE analysis of proteins from *H. annuus* exposed to a mixture of cadmium, nickel, chromium, plus lead (Pb) and lead only.

2.2 MATERIALS AND METHODS

2.2.1 Plant Materials and Metal Treatments

Seeds of the Teddy Bear cultivar of the Dwarf Sunflower (*H. annuus*) were purchased from the Jung Feed Company (Madison, WI). Seven plants were used in each experiment and were grown and treated with metal ions according to the procedures described in January et al. (2008). Briefly, the plants were exposed to heavy metals in solutions containing a mixture of Cd, Cr, Ni (reflecting the contaminated soil conditions in Northeast Ohio) plus Pb or Pb alone. In some cases As was used instead of Pb. The metals were applied as Cd^{2+} ($\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$), Cr^{3+} ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$), Ni^{2+} ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$), Pb^{2+} ($\text{Pb}(\text{NO}_3)_2$) and As as (Na_2HAsO_4) at 30 mg/l for each metal. Seeds of each cultivar were first grown hydroponically in Rockwool, a non-reactive, nonabsorbent support system and sustained with a standard hydroponic nutrient solution (Cropking Corp., Seville, OH). After a four-week growth period, plants were transferred to a

PVC trough and subjected to the contaminant solution at a complete recycle rate of 1.59 l/h containing 30 mg/l of the toxins. After 17 days of exposure, the plants were harvested. Roots, leaves and stems were then sectioned and weighed. All the experiments were repeated twice and the tissues were used for analysis of metal levels and for protein isolation. This part of the work was done at the University of Akron, Department of Civil Engineering. Plant material was shipped to CSU on dry ice and stored at -80°C until use.

2.2.2 Extraction of Soluble Proteins from Leaves

The plant samples were ground in liquid N_2 using a pre-chilled mortar and pestle. The powdered tissue was stored in microcentrifuge tubes (1.5-2.0 ml) at -80°C until further use. A protein extraction kit was purchased from G-Biosciences (St. Louis, MO) and the protein extraction buffer was prepared by dissolving 1 g of dry protein extraction reagent in 1.1 ml of the Diluent which makes approximately 2 ml of the protein extraction buffer. Complete Plant Protease Inhibitor cocktail (G-Biosciences) was added (10 μl into 1 ml protein extraction buffer) and vortexed. Ground tissue powder (approximately 0.2 g per tube) was placed in a microcentrifuge tube and mixed with 500 μl of extraction buffer. The resulting mixture was sonicated six times, ten s each, with one min breaks at 60 rpm in an ice bath and centrifuged at $14,000 \times g$ for 30 min at 4°C . The pellet was resuspended in one-fourth of buffer used in the previous step and sonicated briefly. It was centrifuged again and the supernatants were pooled and stored at -80°C until use.

2.2.3 Protein Assay

Protein concentration was determined using Bio-Rad Protein Assay reagent (Hercules, CA) with 1 mg/ml bovine serum albumin as a standard, according to the manufacturer's protocols. The buffers used in the determination of protein concentrations were different according to the sample preparation methods used. The protein solubilization buffer (G-Biosciences) was used only for the trichloroacetic acid and acetone precipitation. In the case of phenol extraction and PEG fractionation methods, the protein pellet was dissolved in a rehydration buffer (RB) (7 M urea, 2 M thiourea, 4 % NP-40, 50mM DTT, 1% Ampholytes and 0.002% Bromophenolblue). The assay involved addition of Coomassie Brilliant Blue G-250 to the protein extract and absorbance was measured at 595 nm with a Beckman D-64 spectrophotometer.

2.2.4 Protein Extraction for 2-D Electrophoresis

In order to compare the protein extraction efficiency, resolution, and the presence of interfering substances in 2- DE, five protein extraction procedures were tested. In procedure 1, proteins were extracted using the method described by Jacobs et al. (2001) and Damerval et al. (1986). It employs 10% trichloroacetic acid (TCA) containing 0.07% β -mercaptoethanol (2-ME). In procedure 2, proteins were precipitated using only ice-cold acetone/0.07 % 2-ME as the precipitant instead of using 10 % TCA/acetone/0.07% 2-ME mixture. A pellet was obtained using both procedures by 2 h incubation at -20°C followed by centrifugation (14,000 x g, 30 min, 4°C). In procedure 3, which was adapted from Wang et al. (2003), with some

modifications, SDS/phenol was used as the extraction agent. In the phenol extraction procedure, TCA/ Acetone and Acetone washed dry powder was suspended in 0.4 - 0.8 ml/0.1 g of SDS buffer (30% sucrose, 2 % SDS, 0.5 % 2-ME, 0.1 M Tris.HCl, pH 8.0) and Tris-buffered phenol (pH 8.0) in a 1:1 ratio. The mixture was vortexed and centrifuged (14,000 x g, 15 min, 4 °C). The upper phenol phase was collected in a separate microcentrifuge tube and four volumes of 0.1 M ammonium acetate in 80% methanol was added and incubated overnight at -20 °C. The mixture was centrifuged (14,000 x g, 15 min, 4 °C) and the pellet was then washed twice with 0.1 M ammonium acetate in 80 % methanol followed by washing twice with 80% acetone. Finally the pellet was air dried and stored at 80 °C until use. In procedure 4 (double precipitation), the TCA/acetone precipitation method was combined with the SDS/phenol extraction. The TCA/acetone and acetone washed powder was air-dried, mixed with 0.4 - 0.8 ml/0.1 g starting material of 1:1 SDS buffer/ Tris buffered phenol (v/v) and vortexed for 30 s. After centrifugation (16,000 x g, 5 min, 4 °C), the upper phenol phase was mixed with four volumes of 0.1 M ammonium acetate in 80% methanol and incubated overnight at -20 °C. The microcentrifuge tube was centrifuged (14,000 x g, 5 min, 4 °C) and the pellet was then washed once with 0.1 M ammonium acetate in 80 % methanol followed by an acetone washing. The pellet was air-dried and dissolved in 500 µl of 1 % SDS buffer. The protein concentration was determined and 200 µg of protein was re-precipitated with 10 % TCA/acetone/0.07 % 2-ME. After the overnight incubation at -20°C, it was centrifuged (16,000 x g, 4⁰C, 10 min). The pellet was washed twice with ice-cold acetone in 0.07 % 2- ME, air-dried and stored in -80 °C until further use. Finally, another procedure was tested, which employs polyethelene glycol (PEG) fractionation as described by Xi et al. (2006). Some modifications were made in order to minimize loss of proteins as described below. Dry tissue (0.2 g per tube) was homogenized in 1 ml of ice cold

protein extraction buffer (0.5 M Tris.HCl, pH 7.8, 2% (v/v) NP-40, 20 mM MgCl₂, 2% 2-ME (v/v), and Complete Protease Inhibitor cocktail (G-Biosciences), 1 mM EDTA and 1 % (w/v) polyvinylpyrrolidone (PVPP)). The cell free slurry was prepared by sonication and mechanical grinding. It was centrifuged at 12,000 x g, 4 °C for 15 min and the pellet was labeled as *F1*. The supernatant was then subjected to PEG fractionation. To the supernatant, 50 % (w/v) PEG (PEG 4000) stock solution was added to give a final PEG concentration of 16 %. The mixture was incubated in an ice bath for 30 min and centrifuged (12,000 x g, 4 °C, 15 min) and the pellet was labeled as *F2*. The supernatant was mixed with four volumes of cold 10 % TCA/acetone with 0.07 % 2-ME and incubated overnight at -20 °C. After the centrifugation (15,000x g, 4 °C, 10 min) the resulting pellet gave rise to fraction 3 (*F3*). All pellets were washed with ice-cold acetone with 0.07 % 2-ME, vacuum dried and stored at -80 °C until use.

2.2.5 Isoelectric Focusing

Proteins were first separated by isoelectric focusing (IEF) using the ZOOM IPGRunner system (Invitrogen, Carlsbad CA), pH 3-10 non-linear 7 cm IPG strips and rehydration buffer (RB; contained 7 M urea, 2 M thiourea, 4% NP-40, 50 mM DTT, 1 % ampholytes and 0.002% Bromophenol Blue) according to the manufacturer's instructions. Approximately 200 µg of the sample was diluted with RB to give a final volume of 160 µl. Samples were first applied in the mode of in-gel RB of the IPG strips and passive rehydration was carried out overnight at room temperature. Prior to rehydration, 50mM DTT and Bromophenol Blue (3.2µl /160µl RB) were added in to the buffer. IEF was performed at room temperature for 20 min at 200V, 15 min at 450V, 15 min at 750V and 1 h at 2000 V. The total volt-hours applied in the IEF step was 250.

Prior to the second dimension analysis, the strips were equilibrated for 15 min in 10 ml equilibration solution (1 ml of 10 x sample reducing agent mixed with 9 ml of 1 x LDS sample buffer (Invitrogen) and subsequently for another 15 min in 10 ml of 125 mM alkylation solution (232 mg of iodoacetamide in 10 ml of 1x LDS sample buffer).

2.2.6 Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The second dimension (SDS-PAGE) was performed in Nu-PAGE Novex 4-12 % Bis-Tris ZOOM gradient Gel (Invitrogen) with IPG wells following manufacturer's instructions. Protein molecular weight marker (Fermentas, Hanover, MD) was applied to the well provided on the gel for calibration of the molecular weight. The gels were run with an electrophoresis buffer (MES-SDS running buffer; Invitrogen) and electrophoresis was performed at 200 V for 35-40 min. Gels were stained with Simply Blue Safe Stain (Invitrogen) following the manufacturer's protocol.

2.3 RESULTS AND DISCUSSION

2.3.1 TCA-Acetone and Acetone Precipitation

Various conditions of protein extraction affect the number and kind of protein spots after two-dimensional gel electrophoresis, therefore, a good sample preparation is essential for obtaining reproducible 2-D gel results. The effectiveness of two commonly used protein precipitation methods was compared first. These employ trichloroacetic acid (TCA)-acetone

(Jacobs et al., 2001) and acetone alone, respectively. The results of the 2-DE analysis of the TCA-acetone precipitation of proteins obtained from *H. annuus* (Teddy Bear) leaves are shown in Fig. 6. Overall, the number of spots detected in the TCA-acetone treated samples (Fig. 6A) and acetone treated samples (Fig. 6B) were similar. However, some of the protein spots were weaker in the TCA-acetone precipitated samples. This might be due to inability of certain proteins to dissolve, suggesting that some proteins in the samples were susceptible to irreversible denaturation by the acid treatment. Similar observations were previously reported when *Mycorrhiza* were treated in the same way (Hall, 2002). Further, a number of protein spots present in the TCA precipitate were not detectable in the acetone-precipitated samples, but a number of new proteins appeared. This salting out effect of TCA was also observed in the study reported by Jiang et al. (2004) who found that acetone precipitation resulted in an efficient sample concentration and desalting with good recovery of proteins in the 2-DE analysis for human plasma samples. It also is apparent that the leaf samples isolated from *H. annuus* contained materials that cause streaks throughout the gel. The streaks in the molecular mass region of 50-60 kDa were particularly heavy and were always present. The LC-MS-MS analysis indicated that they were due to the presence of RuBisCO.

To minimize the effect of streaking, a number of methods were tried. The phenol extraction method was first described by Hurkman and Tanaka in 1986. Since then sample preparation based on phenol extraction has become the most commonly used protocol in plant proteomic studies (Vincent et al., 2006; Zukas and Breska 2005; Wang et al., 2006), largely because phenol is effective in the removal of interfering compounds like polyphenols. To this end a combination of TCA/acetone precipitation with acetone washes followed by SDS/Phenol extraction was tested. Figure 6C shows the 2-DE of the samples prepared by the phenol

extraction procedure. The results depict a decrease in the number of protein spots, but also a lower amount of streaks as compared to the TCA-acetone or acetone protocols. Furthermore, a number of proteins not previously detected, appeared. This difference could be the result of loss of nucleic acids that also could cause the streaking effect in 2-D gels (Hurkman and Tanaka 1986; Wang et al., 2006).

2.3.2 Double Precipitation

Another protocol tested was the double precipitation method recently described by Gomez-Vidal et al. (2008) to overcome the problem of high background staining. When the 2-D gel obtained using the double precipitation method (Fig. 7A) was compared to either TCA/acetone (Fig. 6A) or acetone precipitation (Fig. 6B), the appearance of streaks or background staining did not change significantly.

In addition, although the overall protein distribution did not change much, a number of proteins present in the acetone or TCA/ acetone protocols were not present when the double precipitation method was used indicating that this protocol resulted in a greater loss of proteins. On the other hand, when the protein pattern for the control samples (Cd, Cr, and Ni present, reflecting the condition of contaminated soils in Northeast Ohio) (Fig. 7A) was compared to Cd, Cr, Ni, plus Pb treated plant sample (Fig. 7B) four extra protein spots (labeled 1-4) were visible. In this regard, the double precipitation method improved over the TCA/acetone or acetone protocol, which only showed 2-3 extra proteins in the all metal treated samples (data not shown). Analysis of these spots will be reported in Chapter 4.

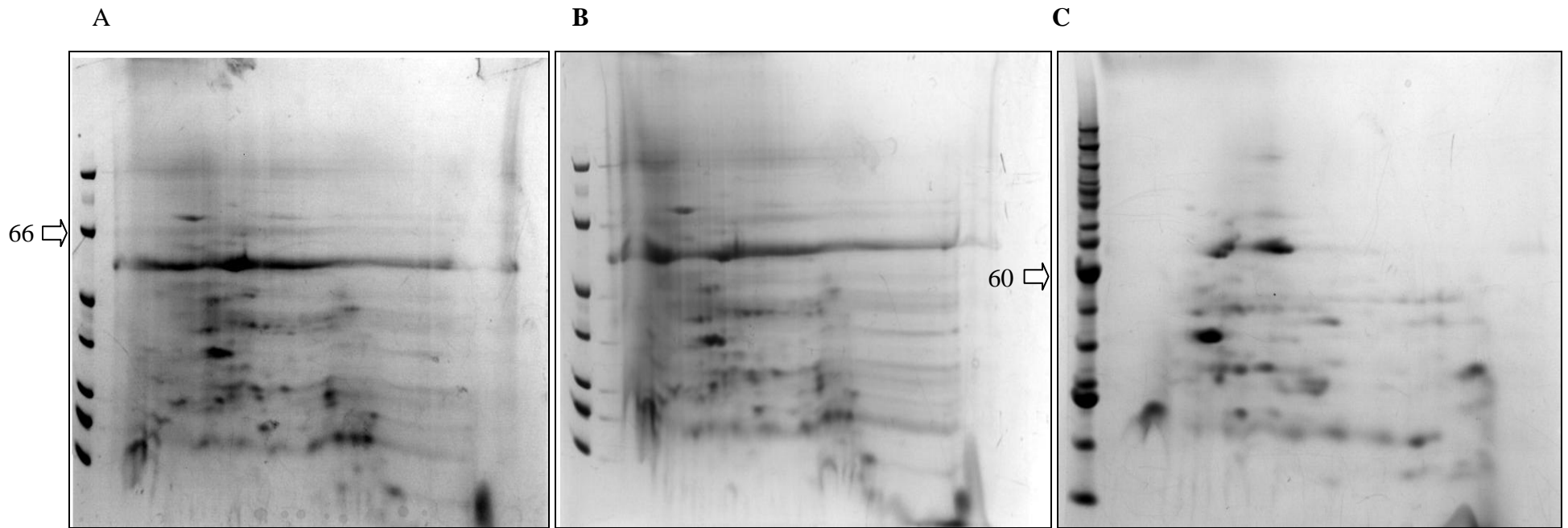


Figure 6: 2-D gel of leaf proteins obtained from *H. annuus* Teddy Bear cultivars.

All proteins were obtained from 150 g of leaves from plants grown under hydroponic conditions. Three sample preparation methods were tested. **A:** 10% TCA/acetone precipitation. **B:** acetone precipitation. **C:** SDS/phenol extraction. Gels were stained with Coomassie Blue. The size of the marker proteins for Gel A and B: 116, 66, 45, 35, 25, 18, 14 kDa and for Gel C: 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 15 and 10 kDa)

2.3.3 Polyethylene Glycol Fractionation (PEG)

The polyethylene glycol fractionation (PEG) method described by Klose (Klose, 1999) for animal tissue was later adapted to plant tissues to overcome the problem of the low concentration of protein in plant tissues and minimizing the loss of proteins. The whole cell protein content made the protein gel more crowded, making identification and analysis of proteins difficult. The PEG-based method is thus intended to improve resolution over the precipitation methods.

More importantly, the PEG fractionation might also reduce the amounts of the highly abundant RuBisCO in the tissues of *H. annuus*. The PEG fractionation described recently by Xi et al. (2006) indicated that RuBisCO might be removed by PEG precipitation. This approach was tested on the Sunflower samples. Using the five-fractionation steps as described, with PEG concentrations up to 24%, it was found that three fractionations minimized the loss of proteins. The 1D PAGE (Fig. 8) indicated that fraction 1 (F1) contained substantially higher levels of RuBisCO than either fraction 2 (F2) or fraction 3 (F3), showing that RuBisCO levels could be reduced by treatment of the sample simply with 16% PEG.

The 2-DE profiles shown in Figure 9 were from Teddy Bear leaf protein samples prepared according to this modified PEG method. Figure 9B shows the samples that were prepared from leaves exposed to the metals Cd, Cr, Ni, and As. Three proteins (labeled 1-3) (Fig. 9A) were visible in the control samples and were absent in the metal-treated samples (Fig. 9B) indicating that these proteins were absent when plants were exposed to all metals. Conversely, six proteins (labeled 4-9) were new in samples from plants exposed to all these metals (Fig. 9B), suggesting that these proteins were up-regulated due to metal-exposure.

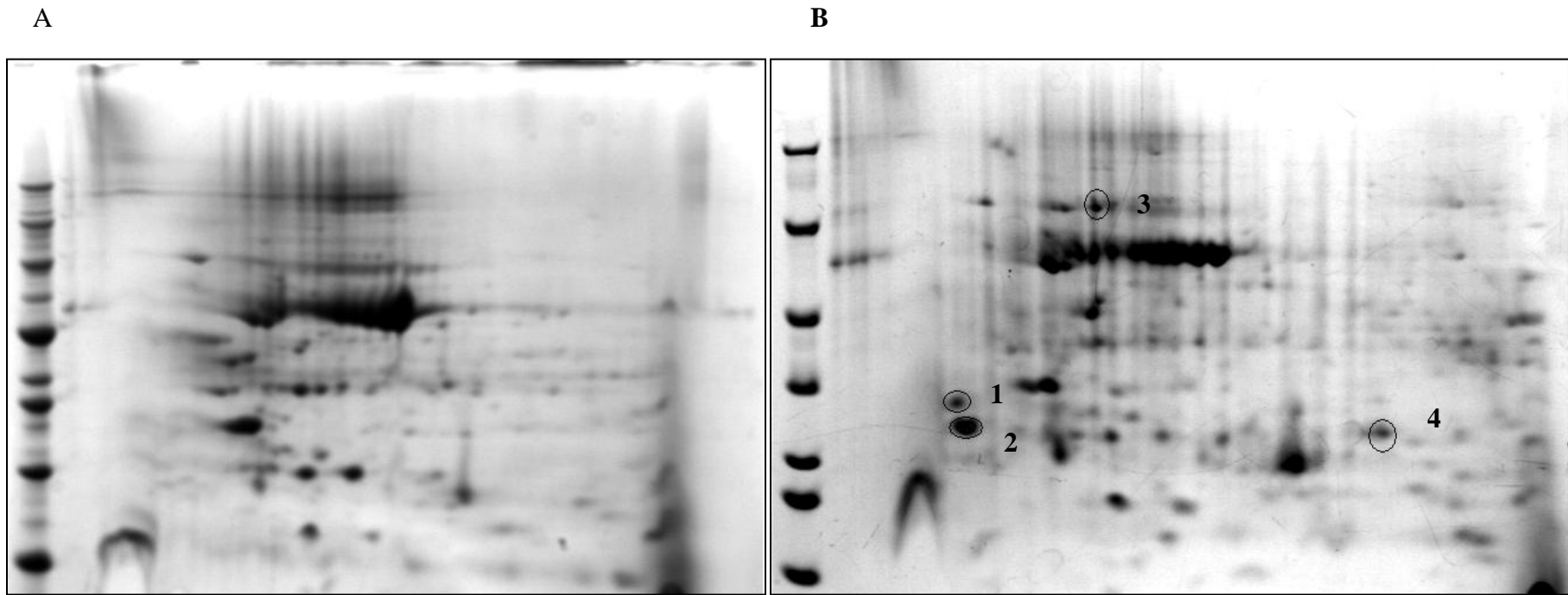


Figure 7: 2-D gel of leaf proteins obtained from *H. annuus* Teddy Bear cultivars using double precipitation

2-D gel obtained following double precipitation on leaf proteins (200 g) of Teddy Bear grown under hydroponic conditions. **A:** Plants served as controls where in they were exposed to Cd, Cr, Ni. **B:** Plants were exposed to Cd, Cr, Ni and Pb. Four (4) proteins were up-regulated upon metal exposure.

Protein Marker for gel A: 150, 100, 75, 50, 35, 25 and 15 kDa and protein marker for gel B: 116, 66, 45, 35, 25, 15 and 10 kDa

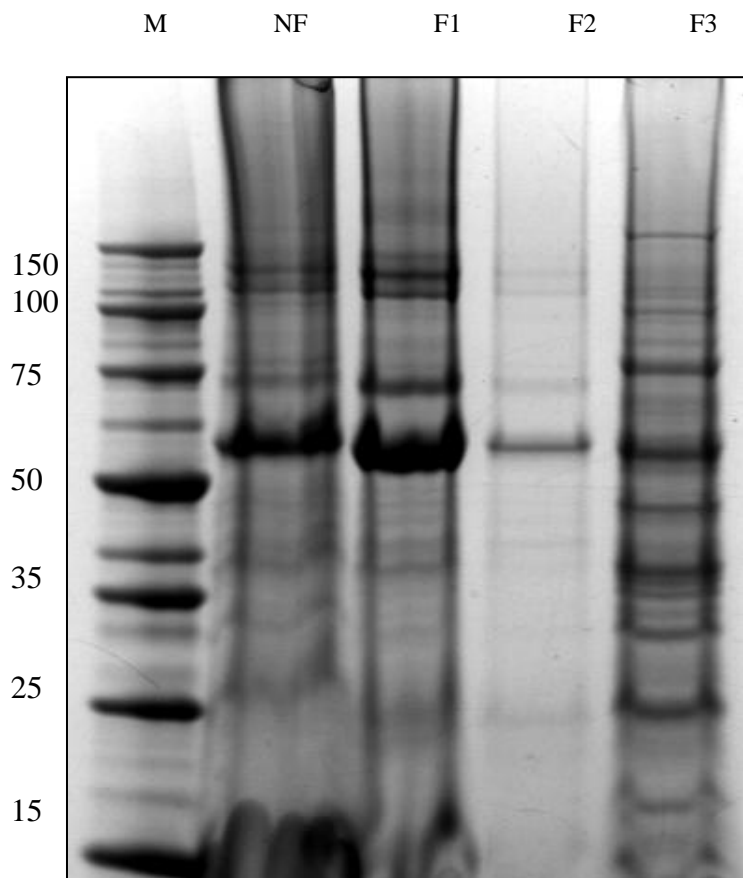


Figure 8: 1-D gel of leaf proteins obtained from *H. annuus* Teddy Bear cultivars using PEG fractionation

SDS-PAGE obtained following the PEG fractionation on leaf proteins of Teddy Bear grown in soil. Plants were not exposed to the test metals. (M = Molecular Marker, NF = whole cell proteins prepared without fractionation, F1 = pellet obtained from cell free slurry after centrifugation (No PEG), F2 = Pellet obtained from 16% PEG, F3 = pellet obtained from the supernatant of F2 treated with 10% TCA/Acetone.

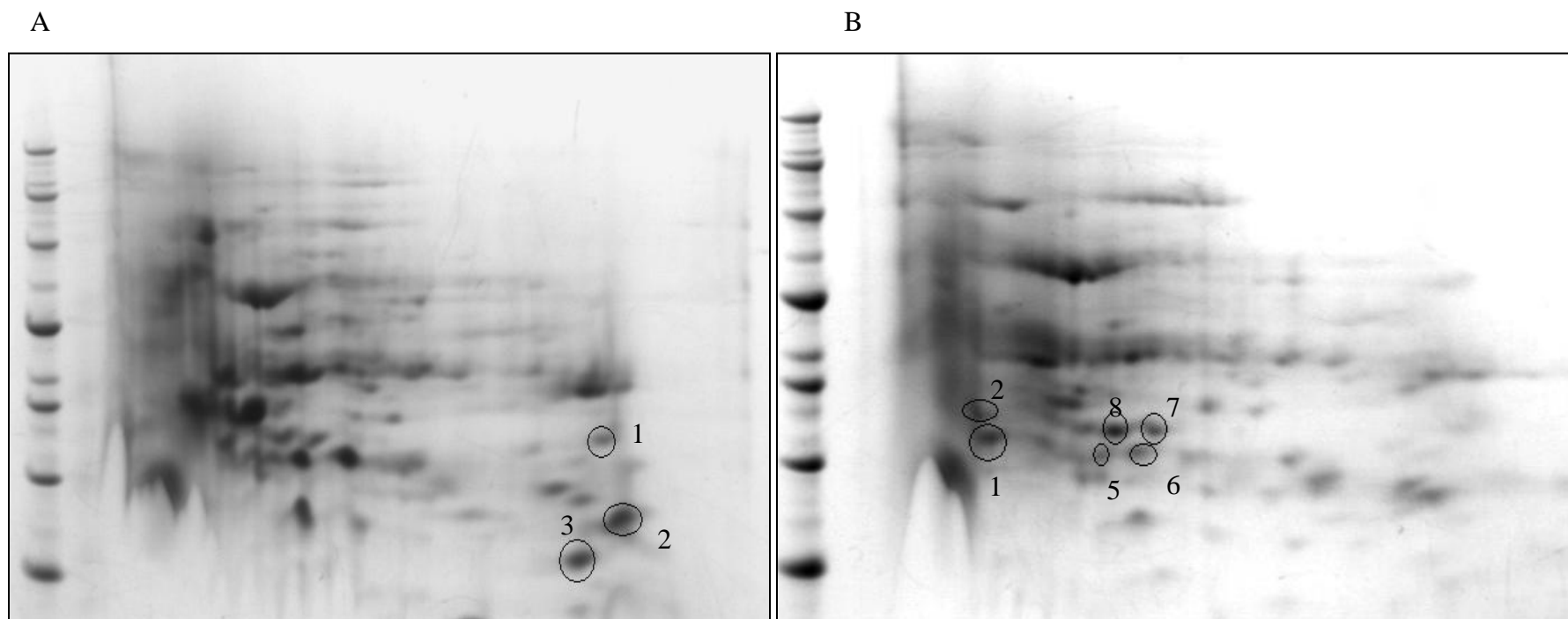


Figure 9: 2-D gel obtained following the PEG fractionation (F3) on leaf proteins of Teddy Bear grown in soil

A: Plants served as controls and spots labeled 1-3 were not observed in metal treated sample in Fig 4B. **B:** Plants were exposed to testing metals Cd, Cr, Ni and As. Spots labeled as 1-6 were not observed in the control sample in Fig. (4A). The protein marker for both gel A and B are 150, 100, 75, 50, 35, 25 and 15 kDa.

2.4 CONCLUSION

Several sample preparation procedures were compared for the Dwarf Sunflower (*H. annuus*), to identify differentially expressed proteins for 2-DE analysis. Use of a phenol extraction method yielded better resolution of proteins compared to either acetone or trichloroacetic acid (TCA/acetone) precipitation techniques. TCA/acetone precipitation in combination with a phenol treatment (double precipitation) further improved gel resolution with regard to reducing background staining and horizontal streaks, however the heavy streaks associated with high concentrations of ribulose 1, 5-bisphosphate carboxylase still persisted. Treatment of the samples with polyethylene glycol (PEG) was simple and effective in reducing this masking protein.

Analysis of the obtained proteins will be presented in the next chapters.

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CHAPTER 3

ARSENATE-INDUCED EXPRESSION OF A CLASS III CHITINASE IN THE DWARF SUNFLOWER *HELIANTHUS ANNUUS*

3.1 INTRODUCTION

Arsenic contamination is widespread as the result of various anthropogenic activities and natural process and the resulting contamination of water and soil poses a major health problem since As is toxic to most living systems including humans, animals, and plants (Fowler, 1983) and this was described in detail in chapter 1. Remediation of arsenic-contaminated soils and water has, therefore, become a major environmental issue and phytoremediation is regarded as a promising approach in finding a solution to this problem (Terry and Banuelos, 2000; Pilon-Smits, 2005).

Since *Helianthus annuus* is reported as a known hyperaccumulator for some metals such as Cr, Ni and Cd, this approach was taken to investigate it's As hyperaccumulation ability and how far it can contribute to elucidate the underlying mechanisms of

hyperaccumulation of metals which is beginning to be understood. In general, the hyperaccumulation of toxic metals is influenced by the efficiency and capacity of uptake, intercellular transportation and sequestration (Yang et al., 2005). Some of the principles governing these processes have been discussed in Chapter 1 such as oxidative stress and involvement of antioxidative defense mechanism, heavy metal chelation by metal binding proteins like PCs and MTs, photosynthetic system damage and its repair as well as chaperone activation. Similarly, chitinases are often expressed as the result of biotic stress such as fungal and bacterial infection, but might have other functions such as the response to abiotic stress by heavy metals (Graham and Sticklen, 1994; Passarinho and de Vries, 2002). Chitinases are a group of enzymes that catalyze the degradation of chitin, a linear β -1-4-linked polymer of *N*-acetyl-d-glucosamine (GlcNAc) and are found in fungi, yeasts, animals as well as in plants; they are divided in two families (18 and 19), and subdivided in five classes.

As an initial approach to using the dwarf sunflower, *Helianthus annuus*, for phytoremediation of metal containing soil the phytoremediation efficiency was investigated when the sunflowers were grown hydroponically and then exposed to various heavy metals, alone or in combination.

The hydroponics experiments and quantification of metal uptake and transport was performed by Dr. Theresa Cutright at the University of Akron.

3.2 MATERIALS AND METHODS

3.2.1. Contaminant Source

Sunflowers were exposed to heavy metals in solutions containing single metals (As, Cd, Cr or Ni) and combinations of Cd plus Cr and Ni, with or without As. Each heavy metal was added at 30 mg/l. The 30 mg/l refers to the concentration of the individual metal, not the compound added. The metals were applied as As^{5+} ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$), Cd^{2+} ($\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$), Cr^{3+} ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$), and Ni^{2+} ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$).

3.2.2. Cultivar Source and Determination of Metals in Plants

Experiments were conducted with two different *Helianthus annuus* cultivars (Sundance and Teddy Bear). Seeds of each cultivar were grown hydroponically in Rockwool, a non-reactive, nonabsorbent support system and sustained with a standard hydroponic nutrient solution (Cropking Corp., Seville, OH). The seeds were grown in a greenhouse illuminated with natural light. The average temperature of the greenhouse was 28 °C (winter) and 35 °C (summer) during the day and 20 °C at night. After a 4 week growth period, seven sunflowers were transferred to a PVC trough. One set of plants was then subjected to a 4 liter solution containing 30 mg/l of the metals as described above. Solutions were introduced at a complete recycle rate of 6 gallons per hour. After 17 days of exposure, the plants were harvested and the experiment repeated with the Teddy Bear cultivar, using fresh solutions. To harvest the plants, the Rockwool was first carefully removed from the roots. Roots, leaves

and stems were then sectioned and weighed. Half of the collected tissue were shipped to be immediately used (i.e., not dried) for protein and RNA isolation (Cleveland State University). The remaining tissue was dried followed by an acid digestion for metal analysis using flame atomic absorption spectroscopy (FAAS) (University of Akron; Buck 200 AA).

3.2.3. Analytical Methods

To determine heavy metal content, the procedure proposed by Zheljzkov and Erickson (1996) was used. One gram of milled plant tissue was soaked in 20 ml of concentrated nitric acid for 6 h. Next, the mixture was concentrated by boiling to 50% of the original volume and then 4 ml of perchloric acid was added and the mixture was refluxed for 90 min. The solution was then diluted with water to a final 20 ml and analyzed by FAAS. The detection limits for the target metals were 0.028 mg/l for Cd, 0.078 mg/l for Cr, 0.14 mg/l for Ni and 0.25 mg/l for As. The concentrations of Cd, Cr, Ni, and As were determined by calibration curves obtained using standards solutions of pure metal ions (Fisher Scientific, Waltham, MA).

3.2.4. Statistical analysis

The results from the four studies were analyzed using MINITAB version 14 software. Experimental results were compared using a general linear model. Statistical significance

was determined using Tukey comparisons with *P*-values <0.05 were considered statistically significant.

3.2.5. Protein Extraction and in-gel Tryptic Digestion

The plant tissues were mashed with mortar and pestle in liquid nitrogen, and 100–200 mg were placed in extraction buffer. The plant extraction solution FocusTM Plant Proteome (G-Bioscience, St. Louis, MO) was used following the manufacturer's instructions, and then centrifuged for 20 min at 15,000 × *g*. The supernatants were collected and the protein concentrations were measured using the Bradford method with albumin as the reference protein solution. SDS-PAGE (Weber and Osborn, 1969) was performed to establish distribution of proteins, molecular mass of the proteins present in the control and the metal-exposed plant biomass. Gels were stained with Coomassie Brilliant Blue. Proteins from stained gels were cut out with a razor blade and treated with the in-gel tryptic digestion kit (Pierce, Rockford, IL) following the manufacturer's instructions. Digestion was conducted overnight at 30 °C.

3.2.6. Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

The digested protein samples were analyzed using an EsquireHCT Bruker Daltonics mass spectrometer equipped with a standard Agilent electrospray (ESI) ion source (Bruker Daltonics, Billerica, MA). Samples were loaded in 10 µl aliquots onto the column. Peptides were enriched using a Peptide C18 CAPTRAP (Michrom Bioresources, Auburn, CA) and

were subsequently separated on a reverse-phase C18MS column (100×0.3 mm, 5µm, 300A; Vydac, Hesperia, CA). Initial Peptide Cap Trap loading was performed using the following conditions: 10 µl/min with a 2% acetonitrile (ACN), 1% formic acid solvent mixture in water for 10 min on a LV-10 ADVP pump (Shimadzu, Columbia, MD). At 10 min loading solvent flow switched to primary LC flow on an Agilent 1100 series (Agilent, Palo Alto, CA). Peptides were resolved using the following conditions: flow rate of 5 µl/min linear gradient from 5 to 40% buffer B (0.1% formic acid, 99.9% ACN) in solvent A (0.1% ACN, 0.1% formic acid in water) for 70 min, then 40–80% B for 30 min. The instrument settings were as follows: nebulizer-11.0 psi, flow rate of dry gas 5.0 l/min; temperature 310 °C; ESI voltage 3.0 kV; scan mode-standard-enhanced with range 50–3000*m/z*, speed 8, 100*m/z/s*; trap settings-positive polarity, ICC Smart Target-105; maximum accumulation time 2×10⁴ ms, scan- 350–1700*m/z*; and target mass-800*m/z*. MS/MS data were acquired using an Auto MSn feature that functions in the “Triple Play” type data-dependent mode, a total scan that included three scan events: a full range MS scan; a narrow range, high resolution zoom scan on a selected ion from the MS scan; and a MS/MS scan of the selected ion. The data were searched against GenBank™ using the MASCOT software.

3.2.7. DNA and RNA Methods

RNA was isolated by modified standard methods. Plant material was ground in liquid nitrogen and then RNA was purified using the Ambion RNA-aqueous mini preparation method using the manufacturer’s protocol (Ambion, Houston, TX). Eluted RNA was pooled

from several columns and ethanol precipitated. Reverse transcription coupled polymerase chain reaction (RT-PCR) was used to amplify cDNA from total RNA (0.5 µg) using a gene specific antisense primer (see below), and Moloney Murine Leukemia virus reverse transcriptase (Epicentre, Madison, WI) according to the manufacturer's protocol. The primers were designed based on homology of the amino acid sequence with TC15508 in the Sunflower Gene Index and were,

sense: 5_TATCCCACAACATGG*GaATt*CACTCATC and

antisense: 5_TCGCT*cg*AGGACTAGTTTATACTGCCT.

The lower case letters stand for mutations introduced to provide restriction enzyme recognition sequences, indicated in italics as *Eco*RI and *Xho*I, resp. The obtained cDNA (10% of the sample) was then subjected to the polymerase chain reaction (PCR) using these gene specific primers and *Taq* polymerase as described (Van Keulen et al., 1998). Semi-quantitative RT-PCR (comparison of 20 and 35 cycles of the PCR reaction) using RNA from control and the appropriate metal-treated plant material was used to investigate whether mRNA was up-regulated. Since primers were designed to amplify the entire open reading frame (ORF), the DNA fragments were immediately eluted from agarose gels, purified and ligated into pGEM-T Easy vector (Promega, Madison, WI) for sequence analysis and subsequently into pGEX-4T-1 (Novagen, Madison, WI) for expression of the protein using *Eco*RI and *Xho*I to insert the DNA fragment in frame with the GST (glutathione-S-transferase) moiety of pGEX-4T-1 as previously described (Lopez et al., 2003). Plasmids were purified by the alkaline miniprep procedure using the Invitrogen plasmid preparation kit (Invitrogen, Carlsbad, CA). The DNA sequence was determined using a Beckman CEQ 8000

Genetic Analysis System. As controls on the semiquantitative RT-PCR, primers were designed based on the sequence of a metallothionein mRNA

Sense: 5'AAAGGGGAATTCATGTCTTGCTGCAACGGAAAG, and

Antisense: 5'CCTTTGTCGACTCAACAGCTGCAGTTGGTTACC.

These were designed based on the sequence of a type 2 metallothionein gene from *Helianthus tuberosus*, GenBank accession number AF467657. DNA was purified using the DNeasy plant genomic DNA purification kit using the manufacturer's instructions (Qiagen, La Jolla, CA). The same primers as described above were used for amplification of genomic DNA, which was cloned into the pGEM-T Easy vector and sequenced.

3.2.8. Antiserum Production

Antiserum was prepared in two rabbits against a synthetic peptide located at the C-end of the sunflower chitinase with sequence: CRFYDKQSGYSDAIK and attached to Keyhole Limpet protein (Sigma-Genosys, The Woodlands, TX). Western blot analysis was as described (Lopez et al., 2003) using a 1:750 dilution of the antiserum in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 150 mM NaCl) with 0.5% Tween-20 (TBST) and a 2 h incubation at room temperature. A goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase was used and the signal detected with SuperSignal West Pico (Pierce).

3.3 RESULTS

3.3.1. Metal Uptake

Analysis of metal distribution indicated that more metals were sequestered in roots than either stems or leaves under the experimental conditions (Fig. 10). When As was the only contaminant, the root, leaves and stems concentrations were 1.52, 0.52 and 1.04 mg As/g biomass, respectively with no statistical difference in sequestration location. When Cd, Cr, and Ni were also present, As concentration was still highest in the roots at 1.54 mg/g biomass. There was no difference between the arsenic concentration in the leaves (0.56 mg/g) or stems (0.79 mg/g). The presence of the other metals did not appear to alter the As uptake. It is also apparent that uptake of Cr and Cd (around 11 and 3.5 mg/g biomass) were significantly greater than Ni or As (2.5 and 1.5 mg/g biomass). These results suggested that the process of accumulating arsenic in *H. annuus* is governed by mechanisms that are distinct from those of other metals.

Similar studies were performed with the Teddy Bear cultivar. As shown in Fig. 11, the uptake patterns in this strain resembled those of Sundance in that more metals were sequestered in the roots than other tissues. In addition, Cr levels, when compared to other metals, were higher. However, the degree of metal accumulation in Teddy Bear differed with that of Sundance. For instance, the metal uptake in increasing order was Cr > Ni > As > Cd in Teddy Bear whereas the order in Sundance was Cr > Cd > Ni > As. See Table V for a summary of the obtained results.

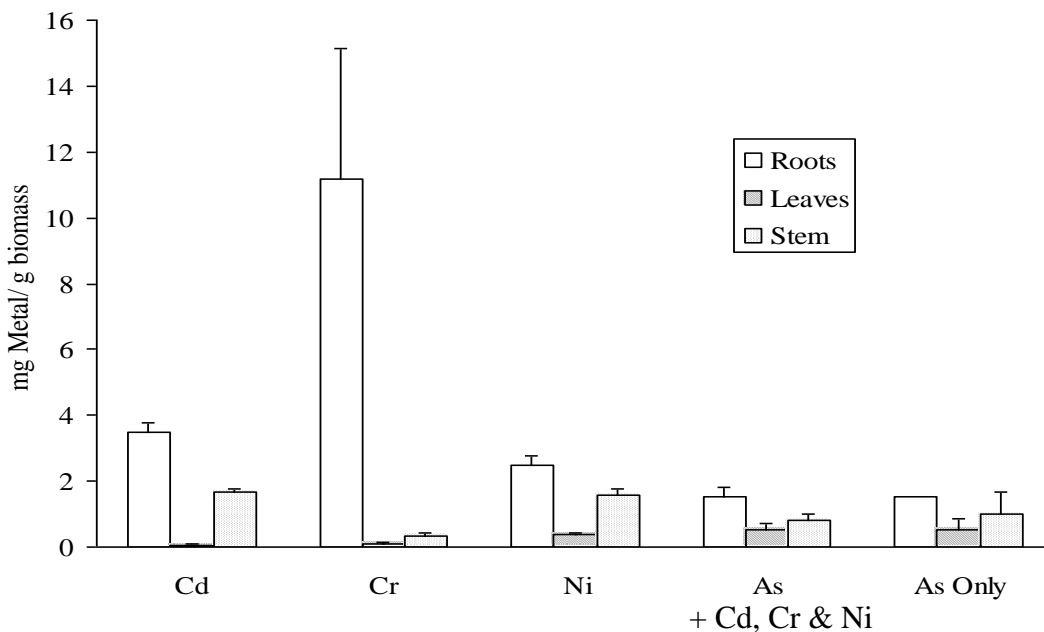


Figure 10: Metal concentration in biomass for Sundance sunflowers

Plants were grown hydroponically and exposed to 30 mg/l As and to 30 mg/l each Cd, Cr, Ni, As. Error bars show variation between replicates. All differences in sequestration location are significant with the exception of As (mixed) leaves-stems, Cr leaves-stems and As only roots-leaves-stems.

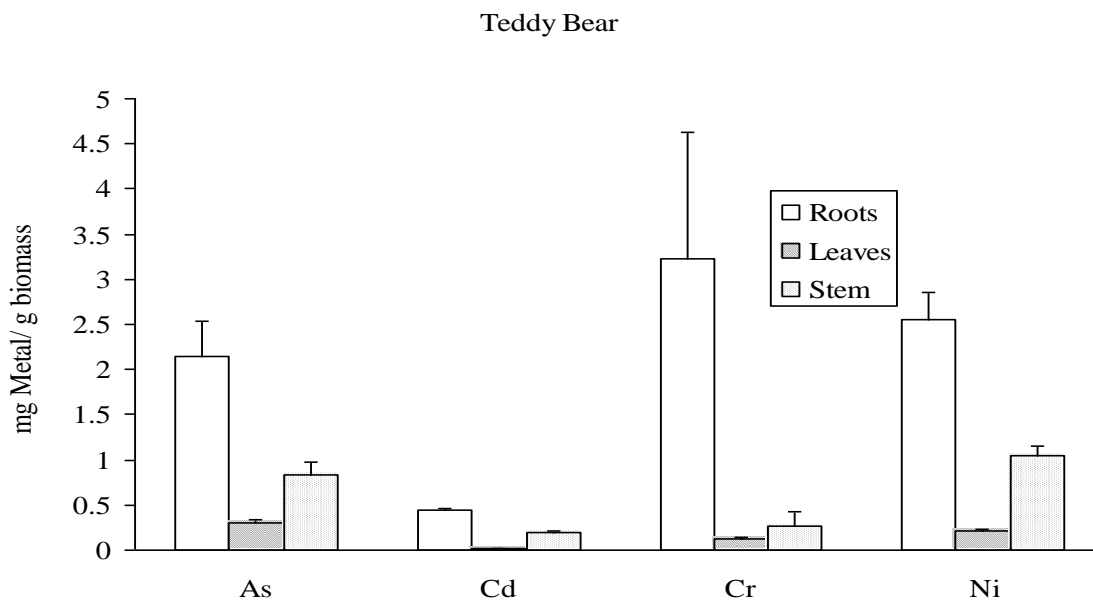


Figure 11: Metal concentration in biomass for Teddy Bear Sunflowers

Plants were grown hydroponically and exposed to 30 mg/l each As, Cd, Cr, Ni. Error bars show variation between replicates. All differences in sequestration location are significant with the exception of Cd leaves-stems and Cr leaves-stems.

Table V: Total metal uptake, biomass and translocation factors for Hydroponic Sundance and Teddy Bear Sunflowers.

Cultivar		Total	Metals (mg)				Total
		Biomass g	As	Cd	Cr	Ni	Metal
Sundance	As Cd Ni Cr	2.682	1.95	2.40	3.92	2.55	10.82
	As Only	2.129	1.59				1.59
Teddy Bear	As Cd Ni Cr	4.757	3.34	0.57	3.00	3.59	10.50
	Translocation Factors (TF)						
Sundance	As Cd Ni Cr		0.4	0.16	0.02	0.3	
	As Only		0.44				
Teddy Bear	As Cd Ni Cr		0.20	0.13	0.05	0.17	

3.3.2. Expression of Proteins in Response to Arsenic

Although roots contained the highest amount of metal, it would eventually be translocated and sequestered in leaves. Therefore, protein was first isolated from leaf material, which also provided more material to analyze. Protein extracts of leaves from Sundance and Teddy Bear were analyzed by SDS-PAGE. Leaves were used from plants exposed to solutions containing all four metals (Cd, Cr, Ni and As), arsenic alone, or three metals (Cd, Cr, and Ni). Control leaves were from plants grown in the absence of metals. Gel electrophoresis analysis (Fig. 12) revealed the presence of several extra polypeptides in the leaf extracts from plants (Sundance) treated with all four metals (lane 3) or arsenic alone

(lane 4) as compared to leaf extracts from control plants (lane 2). Since these polypeptides were not apparent in either the control or leaf extracts from plants exposed to three metals (Cd, Cr and Ni), it was concluded that the expression of these proteins was due to the stress imposed by arsenic. The staining intensity of one polypeptide with an approximate molecular mass of 32 kDa was very strong in these samples. Therefore, this polypeptide was chosen for further analysis. Leaf extracts of Teddy Bear also showed a 32 kDa band only when the plants were exposed to arsenic alone or together with the other metals (data not shown).

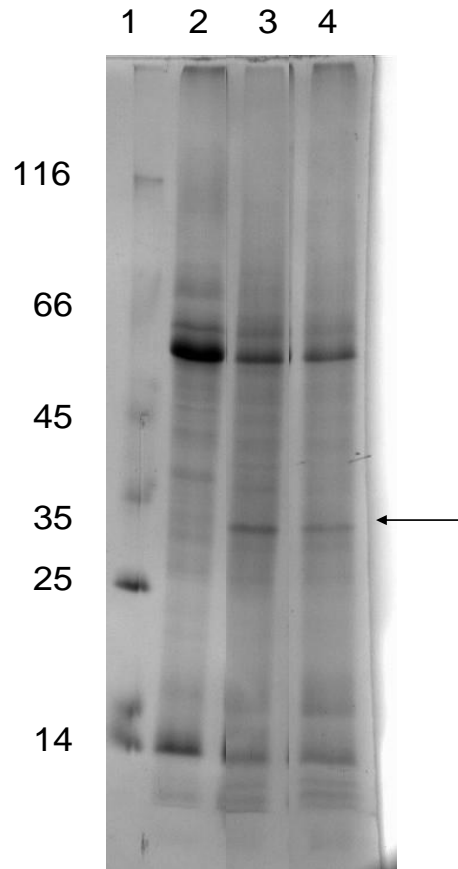


Figure 12: Soluble proteins associated with leaves of Sundance Sunflowers

Proteins were resolved on a 16% SDS-polyacrylamide gel and Coomassie brilliant blue stained. Lane 1: protein size markers; lane 2: control leaf extracts; lane 3: leaf extracts from plants exposed to Cd, Ni, Cr and As; lane 4: leaf extracts from plants exposed to As only. The arrow indicates the 32 kDa polypeptide identified as chitinase.

Table VI: Sundance control and Sample Digests
A. Sundance Sunflower control digest

Match to RuBisCO (*H. annuus*)

Observed	Mr (expected)	Mr (calculated)	Delta	Score	Peptide
369.22	736.43	736.38	0.05	43	R.GGLDFTK.D
511.28	1020.55	1020.52	0.02	73	K.DTDILAAFR.V
530.35	1058.69	1058.55	0.13	30	R.VALEACVQAR.N
593.84	1185.67	1186.66	-0.99	70	R.DNGLLLHIHR.A
615.86	1229.71	1229.63	0.08	86	R.DLATEGNEIIR.E
638.41	1274.81	1274.72	0.08	64	R.EITLGFVDLLR.D
704.31	1406.61	1406.66	-0.06	39	K.LTYYTPEYETK.D
726.81	1451.61	1451.72	-0.11	77	K.TFDGPPHGIQVER.D

B. Sundance AS exposed sample digest

Match to Chitinase from *Cicer arietinum*

Observed	Mr (expected)	Mr (calculated)	Delta	Score	Peptide
548.78	1095.55	1095.52	0.03	72	K.YGGVMIWDR.F

Match to Chitinase from *Trifolium repens*

Observed	Mr (expected)	Mr (calculated)	Delta	Score	Peptide
548.78	1095.55	1095.52	0.03	72	K.YGGVMIWDR.F

Match to Chitinase from *Phaseolus angularis*

Observed	Mr (expected)	Mr (calculated)	Delta	Score	Peptide
548.78	1095.55	1095.52	0.03	72	K.YGGVMIWDR.F

Amino acid sequences of trypsin-treated RuBisCO and 32 kDa protein bands.

A. alignment of RuBisCO protein

B. alignment of 32 kDa protein

3.3.3. Identification of the 32 kDa protein band by LC–MS/MS

The 32 kDa polypeptide was excised from the gel shown in Fig. 12 and subjected to tryptic digestion followed by amino acid sequence analysis by LC–MS/MS. In addition, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) of about 55 kDa, which was present at high levels in leaf protein samples as an intense stained band, was

treated the same way and was routinely used for quality control purposes, see Table VIA. The amino acid sequence of a tryptic peptide obtained from the 32 kDa polypeptide, YGGVMIWDR, shared its sequence with plant chitinases from three species including *Cicer arietinum* (Table VIB). These results suggested that the 32 kDa protein might be plant chitinase. It also matched one of the chitinase sequences in the sunflower gene index, namely TC15508 (The Computational Biology and Functional Genomics Laboratory, The Gene Index Databases, Dana Farber Cancer Institute, Boston, MA, URL:<http://www.danafarber.org/>).

3.3.4. Cloning and Expression of Chitinase

The sequence of TC15508 was used to design primers that cover the start and stop codons of the protein. The primers were designed with mismatches in order to provide restriction enzyme sites to clone the ORF in frame with the GST portion of the expression vector pGEX-4T-1 to be able to express the enzyme for further studies. To establish that this chitinase was up-regulated in As-treated plants, RT-PCR was performed first on identical amounts of RNA isolated from control leaves, leaves from plants exposed to Cd alone and leaves from plants exposed to all metals including As, using the chitinase-specific primers. When all metals were used in the treatment, a chitinase specific cDNA of 1000 basepair (bp) could be detected after 20 cycles (Fig. 13B, lane 6) reaching a high intensity at 30 cycles (Fig. 13B, lane 7). No chitinase-specific product could be identified in samples obtained from Cd-treated plants and a low amount was present in control plants, showing the 1000 bp band only after 30 cycles, but not after 20 cycles. As a control on DNA contamination (see below,

the chitinase gene has no introns) and amount of RNA present in the reactions, the same amounts of these RNA samples were subjected to RT-PCR using metallothionein-specific primers (Fig. 13A).

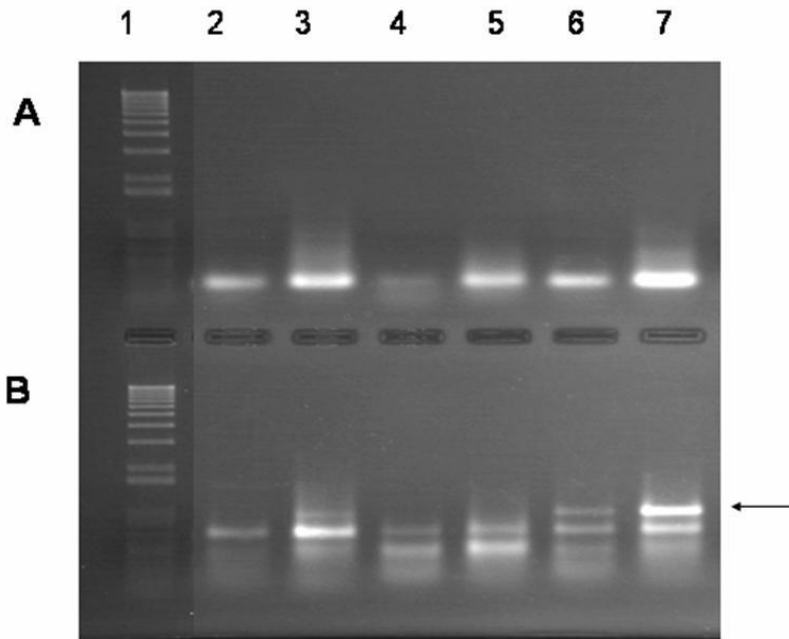


Figure 13: Semi-quantitative RT-PCR of total RNA samples

Reverse transcription was performed on 5 μ g total RNA isolated from leaves as indicated using specific anti-sense primers. Of each reaction 10% of the product was subjected to PCR using 20 and 30 cycles for each cDNA using gene specific primers. Each reaction was size fractionated on a 1.2% agarose gel and stained with ethidium bromide. (A) Control reactions using metallothionein specific primers.

(B) Reactions using chitinase specific primers. Lanes 1: DNA size marker, lanes 2–7: RT-PCR using RNA isolated from different leaf samples of Sundance, lane 2: control 20 cycles; lane 3: control 30 cycles; lane 4: Cd-exposed 20 cycles; lane 5: Cd-exposed 30 cycles; lane 6: all metals +As 20 cycles; lane 7: all metals +As 30 cycles. The arrow indicates full length chitinase DNA.

The type 2 metallothionein gene is 961 bp in size and contains two introns, while the mRNA is only 230 nucleotides (nt). The RT-PCR reaction showed the absence of DNA in

these samples because no band in the 960 range was observed. The presence of similar amounts of a 230 bp RT-PCR product indicated that similar amounts of RNA were used in the assay, although the amount of metallothionein mRNA appeared to be higher in samples obtained from As-treated plants. This could be the result of up-regulation of the metallothionein expression. Use of primers specific for rRNA confirmed that similar amounts of RNA were used in all reactions (data not shown). The 1 kbp DNA that was found to be up-regulated was excised, cloned and sequenced and was identified as a chitinase. The derived protein sequence consists of 303 amino acid residues and the sequence identity was 90% as compared to TC15508. Genomic DNA was also amplified with the same primers and resulted in a similar 1 kbp DNA, which upon cloning and sequencing showed the same DNA sequence, indicating that this chitinase gene has no introns. The sequences are presented in Figure 14. The same approach was used for the metallothionein control, showing that the genomic DNA was indeed 961 bp and has two introns, the resulting metallothionein mRNA was 230 nt and is shown in Figure 15. It has 98% identity with a type 2 metallothionein from *Helianthus tuberosus*. The sunflower chitinase amino acid sequence was aligned with those from *Arabidopsis*. The highest degree of identity was obtained with the *Arabidopsis* gene from locus At5g24090 (Gen-Bank accession number BAA21861.1) with 52% identity. This chitinase has been identified as a class III chitinase. The amino acid sequence of the sunflower chitinase contains the conserved sequence (LIVMFY)-(DN)-G-(LIVMF)-(DN)-LIVMF)-(DN)-x-E (position 145–153: LDGIDFDIE), which is characteristic of family 18, class III of acidic chitinases (EC 3.2.1.14) (Passarinho and de Vries, 2002), see figure 14. The sequence data were submitted to GenBankTM and are available as: EF431954 for metallothionein and EF431955 for chitinase. The chitinase gene was cloned in the expression

vector pGEX-4T-1, but was recovered as an insoluble inclusion body, which could not be used for enzyme analysis. However, the antiserum made against the C-terminal peptide recognized the fusion protein proving its value to detect the enzyme (data not shown).

Genomic and cDNA Sequence

*TATCCCACAA CATG**GAATTC** ACTCATCCAG CACTTCTCCT GCTCCTCTTC ATCACTGTTT*
TCTCTTTTTT GAAACCCTCT ACAGCCGCCG GCATCGCCAC TTACTGGGGC CAGCAGAGCG
ATGATACTGA AGGCACCCTT GAAGCCGCCT GTGCCACCGG AAACCTACCAG TTTGTAAACA
TAGCTTTTCTT AAGCACCTTC GGCAACAACC AACAACCGGT TCTGAACCTA GCAGCCCATT
GTGATCCTGC TTCGACTTGC TCCAGGTACA GCTCCCAGAT AAAAGCATGC CAGGCCCAAA
ATGTTAAGGT GTTTCTTTCT ATAGGGGGTC AGAGAGGGAG CTACTCTCTA TCGTCACCGC
AAGATGCACA ACAAGTTGCT GATTTTCTAT GGAATACCTA TCTTGGTGGC CAGCCAGCTA
CCCGTCCGTT AGGAGATGCT GTTTTGGACG GAATCGATTT TGATATCGAG CAAGGGACAG
ACCAGTTCTG GTCTGACCTG GCTAAGGCAC CCGCTGCGTA CAGCTCACAG AAAAAAGTGT
ACTTATCTGC AGCCCCACAG TGCCCATTC CGAGTGGCGA CGTCCGCAAC CAACTGCTCC
CGGCGATTAG GGAAGGGCTG TTTGATTACG TGTGGGTGCA GTTTTACAAC AATGAACAAT
GCCAGTATGG GGCGAATGCT GATGCTTTAT TAGCGAGATG GAACGAGTGG ACTCAGGTGA
CCACAAATAC GATTTTCTTG GGGTTACCAG CAGCAGCTTC GGGAGCTGCC CCAAGCGGAG
GATATATTCC ACCAGATATT CTTACCTCTC AGATTCTTCC TTCAATCAAG AGTTCTCCCA
AGTACGGTGG AGTCATGCTG TGGGACAGGT TCTATGATAA ACAGAGTGGA TACAGTGACG
*CCATCAAAGG CAGTATAAAC TAGT**CTCGA** GCGA*

Translated Sequence

MEFTHPALLL LLFITVFSFL KPSTAAGIAT YWQQSDDTE GTLEAACATG NYQFVNIAFL
STFGNNQQPV LNLAHCDPA STCSRYSSQI KACQAQNVKV FLSIGGQRGS YSLSSPQDAQ
QVADFLWNTY LGGQPATRPL GDAV**LDGIDF** **DI**EQGTQFW SDLAKAPAAY SSQKKVYLSA
APQCPFPSGD VRNQLLPAIR EGLFDYVWVQ FYNNEQCQYG ANADALLARW NEWTQVTTNT
IFLGLPAAAS GAAPSGGYIP PDILTSQILP SIKSSPKYGG VMLWDR**FYDK** **QSGYSDAIKG** SIN

The primers used for DNA and cDNA amplification are indicated and shown in italics, the restriction enzyme sites for cloning into an expression vector are indicated in bold and underlined (*Eco*RI and *Xho*I). The peptide used to make anti-serum is indicated in red. The class III specific signature sequence is indicated in green. The sequences are presented in blocks of ten nucleotides and amino acids

Figure 14: Sundance chitinase sequences

cDNA Sequence

AAAGGG**GAAT** ***TC**ATGTCTTG CTGCAACGGA AAGTGCGGGT GCGGCTCAAG CTGCTCATGC*
GGCAGCGGCT GCAACGGATG CGGAATGTAC CCTGATGTTG AGGTTTCGTC CACCACCGTC
ATGATCGTTG ACGGTGTTGC CCCAAAACAG ATGTTTGCTG AGGGAAGTGA GGGCAGCTTT
GTTGCTGAGG GTGGAAACTG CAACTGCAAG TGTGGTAACC AACTGCAGCT ***GTTGAGT**CGA***** **CAAAGG**

Translated Sequence

MS**CC**NGK**CGC** GSS**CS**CGSGC NGCGMYPDVE VSSTTVMIVD GVAPKQMF AE GSEGSFVAEG
GNCN**CK**CGNQ LQLLSRQR

The primers used for DNA and cDNA amplification are indicated and shown in italics, the restriction enzyme sites for cloning into an expression vector are indicated in bold and underlined (*Eco*RI and *Sal*I). The cys residues in the two metal binding domains are indicated in red.

Figure 15: Sundance Metallothionein

3.3.5. Expression of chitinase under various conditions

Western blot analysis was performed using the antiserum generated against a C-terminal peptide from the obtained chitinase sequence. Leaf protein extracts were prepared from control Sundance and plants treated with all metals without As and all metals plus As and As alone. In addition, extracts from Teddy Bear using various metal ions (Ni alone, Cd alone, Cr alone and all plus As) were screened in the same manner. The result of a Western Blot screened with the anti-chitinase serum is shown in Fig. 16.

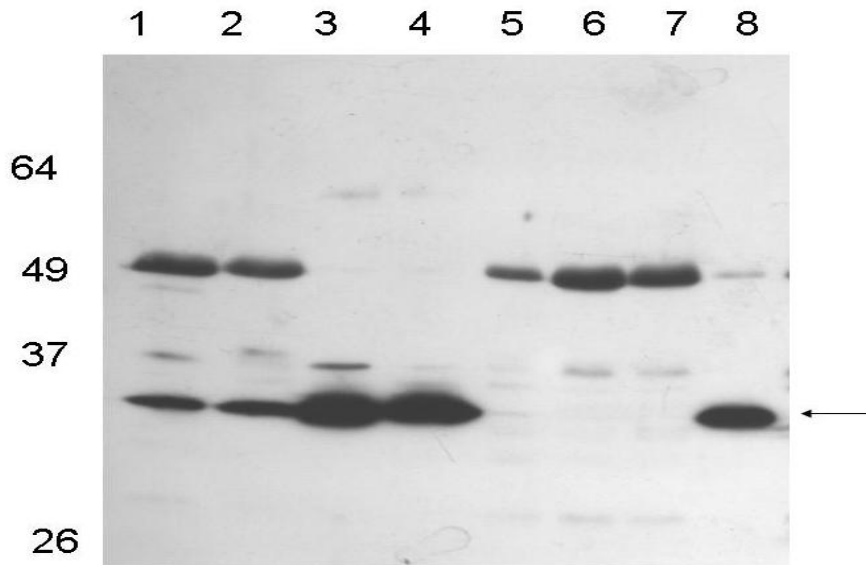


Figure 16: Western Blot analysis of leaf proteins.

Extracts of Sundance, lanes 1–4, and Teddy Bear leaves, lanes 5–8, were separated by SDS-PAGE (38 μ g protein per lane), transferred to a PVDF membrane and exposed to a rabbit anti-chitinase serum diluted 1:750 in TBST. The signal was produced using a goat anti-rabbit secondary antibody conjugated with horse-radish peroxidase. The blots were exposed to X-ray film after treatment of the membranes with SuperSignal West Pico. The arrow indicates the 32 kDa chitinase.

The same amount of protein was loaded in each lane and the results show clearly an increase in a 32 kDa protein, presumably chitinase, when As is present in the Sundance-derived samples (lane 3, all metals plus As; lane 4, As alone). As observed in the RT-PCR experiment some mRNA is present in control plants, and a lower amount of the 32 kDa protein can be detected as well. However, the Teddy Bear samples showed that the 32 kDa protein was only expressed when As was in the medium (Fig. 16, lane 8). Under different conditions, namely other metals but no As, no 32 kDa-specific protein was visible (Fig. 16, lanes 4–7). Another, unknown protein of about 50 kDa showed the opposite expression

profile. It was present in the protein samples as long as As was not present in the growth medium and could also be detected with the pre-immune serum.

Plants were grown hydroponically and in soil and exposed to all metals (Cr, Cd, Ni) with or without As. The same concentration of metals was used in hydroponics and soil-based experiments, except the soil samples had no EDTA added or 0.1 or 0.3 M EDTA to increase metal mobility (Chen and Cutright, 2001). For soil-based experiments, each species was initially sown in commercial potting soil in a greenhouse with a 15 h photo-period. After 4 weeks, seedlings were transferred to the metal spiked soil to initiate the experiment. Three seedlings were used per 0.5 kg soil, for a total of nine per treatment condition. Plants were harvested after 4 weeks. Roots were rinsed with 25 ml distilled water to ascertain if the metals were simply loosely bound to the external wall or had crossed through the root.

Stem, roots and leaves were used for protein extraction. As shown above, chitinase is a good indicator for the presence of As and because the serum only showed a signal with As and hardly any cross-reactivity with other plant proteins on Western blots, the method of dot blotting was tested as a fast way to measure As-related stress in both soil-grown plants and hydroponically-grown plants. This method is especially useful when small amounts of proteins are available such as isolated from stems and roots. Figure 17 shows that hydroponically-grown Sundance expresses chitinase only in the presence of As, as already shown by Western blot analysis. The results show that soil-grown plants do not express chitinase in their leaves, but low levels appear when EDTA is added. A very high expression of chitinase was found in the roots of Sundance grown in the presence of As. Thus chitinase is a good indicator of As-induced stress, especially in the roots.

3.3.6. Use of Chitinase as a tool to detect As in the soil.

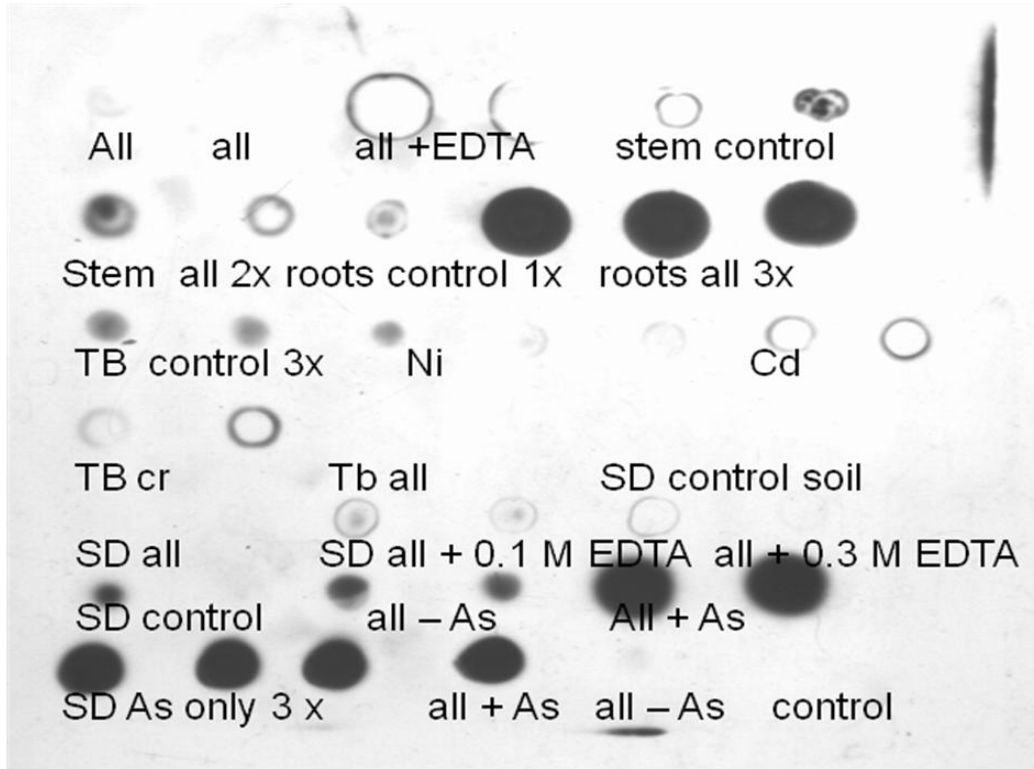


Figure 17: Dot blot analysis of protein samples obtained from Sundance (SD) and Teddy Bear (TB)

Plants were treated with different combinations of metals and proteins were purified. Four μg protein was loaded per dot. The blot was developed with a 1/750 dilution of the anti-chitinase serum and treated as in the legend of Figure 5. Row 1 – 5: proteins from soil-grown plants. Row 6 and 7 from hydroponically-grown plants.

Row 1: All, soil-grown plants with all metals no EDTA added, in duplicate; All + EDTA, same but EDTA present in the soil, in duplicate. From stems only, control: control plants, in duplicate.

Row 2: Stems from plants exposed to all metals, in duplicate; roots: from control plants, one dot, then roots from treated plants, 3 dots.

Row 3: TB control, in triplicate from control plants, Ni-treated TB, in duplicate, Cd-treated TB, in duplicate.

Row 4: Cr-treated TB in duplicate; TB all metals, in duplicate; SD control, in duplicate

Row 5: SD all metals, induplicate, SD all metals + with 0.1 M EDTA in the soil, in duplicate; SD all metals with 0.3 M EDTA in the soil, in duplicate.

Row 6: SD control, Control samples, in duplicate; SD all –As; all metals except As, in duplicate; All + As, all metals + As in duplicate.

Row 7: SD As only; samples from SD exposed to As only, in triplicate. All + As, SD all metals + As. Last three dots: controls, SD + all metals + As, SD + all metal – AS and SD control.

3.4. DISCUSSION

3.4.1. Uptake of Metals

A few plants have been identified as arsenic hyperaccumulating including *Holcus lanatus* (Meharg and Macnair, 1992) and Ladder brake (*P. vittata*) (Cong and Ma, 2002). *Helianthus annuus* has also demonstrated the ability to hyperaccumulate heavy metals such as Cd, Cr, and Ni and Zn (Chen and Cutright, 2001; Lesage et al., 2005). The results of this study showed that *H. annuus* is capable of hyperaccumulating Cd, As and some Ni. However, more was accumulated in the roots and stems than in leaves. Previous studies indicated that the bioavailability and uptake of metals by *H. annuus* was influenced by secretion of phytochelatins and/or low molecular weight organic acids (Lesage et al., 2005). In the case of As, it was observed that arsenate reductase and γ -glutamyl-cysteine synthase were necessary for uptake in *P. vittata*. In another study, As was stored as As(III) tris-thiolate in the shoots of *Brassica juncea* (Alkorta et al., 2004). It is generally believed that the reduction of arsenic from arsenate to arsenite is a critical mechanism for tolerance and subsequent accumulation in plant tissues (Wei and Chen, 2006). Other researchers have reported that arsenate is not taken up via the cation channels. For instance, arsenate was taken up in *Arabidopsis* by phosphate transporters (Wang et al., 2002). Therefore, one adapted mechanism for hyperaccumulating As appears to be to suppress uptake of phosphate via the phosphate transport systems in the roots (Meharg and Macnair, 1992).

Further, it was shown that as an increase of phosphate supply decreased arsenate uptake markedly increased (Wang et al., 2002), thus arsenate appears to compete with

phosphate for the phosphate transporter. This study was performed to further explore the mechanisms of hyperaccumulation of arsenic in *H. annuus*.

3.4.2. Responses to Arsenic Exposure

In response to arsenic exposure, the Sundance cultivar showed expression of a number of proteins. One protein with a molecular mass of 32 kDa was particularly distinct. A similar analysis of the leaf samples from another cultivar, Teddy Bear, also revealed expression of this protein of 32 kDa. This was shown by Western blot analysis using antiserum prepared against this Sundance protein. The Sundance polypeptide was by micro-sequence analysis identified as an acidic endo-chitinase. Although only one tryptic peptide was obtained, identification of this protein as a chitinase was proven by semi-quantitative RT-PCR using RNA isolated from leaves of control and As treated samples using chitinase-specific primers. Because the mRNA appeared to be transcribed from an intron-free gene, the absence of possible contaminating DNA in the RNA samples was shown by using for the same assay an intron-containing gene, a type 2 metallothionein. RT-PCR showed clearly the absence of DNA in these RNA samples and the data suggest that also this metallothionein gene expression might be up-regulated in the presence of As. This result and implication will be addressed separately. Final proof that indeed this protein is a chitinase was provided by Western blot analysis using a rabbit anti-chitinase antiserum, produced against a C-terminal peptide identified in the chitinase sequence. This antiserum, which detected the expressed GST-fusion protein, clearly showed the presence of a 32 kDa protein in both Sundance and Teddy Bear samples, which is specifically up-regulated when As was added to the plants,

alone or in combination with other metals. Apparently sunflowers express this type of enzyme under certain stress conditions, such as As exposure, as shown here. What the actual function is of this protein remains unclear, but the notion that chitinases might have a multifunctional, and in part unknown, role is evident. One possibility is that it is involved in cell death. As shown in Fig. 12, treatment with As lowers the amount of the large subunit of RuBisCO. Further work will be needed to elucidate the role of this enzyme during As-induced stress.

SDS-PAGE analysis also revealed a number of other protein bands in leaves of these plants in response to arsenic exposure, but their identity has yet to be established. Some low molecular weight peptides of less than 10 kDa were observed. These might belong to the phytochelatin family which have molecular masses of 2500 or 3600 Da (Grille et al., 1985) or metallothioneins which have molecular masses in the 7 kDa range. The RT-PCR experiment suggested that metallothionein expression is up-regulated. This will be pursued further as well as the identity of other apparently up-regulated proteins. Finally, with the availability of specific antiserum, the presence and location of this chitinase can now be followed in roots and stems. This was tested using dot-blot analysis with leaf, stem and root proteins. In the presence of As chitinase is expressed strongly in the roots of soil-grown plants while leaves and stem do not show any expression. The hydroponically-grown plants show a much higher response to As in the leaves. This suggests that entry and transport of As is much better in hydroponically grown plants.

3.5. CONCLUSION

The dwarf sunflower cultivars Sundance and Teddy Bear accumulate several heavy metals simultaneously. In the presence of As alone or As plus other metal ions it was found that a chitinase gene was induced specifically and strongly at the transcription level. This chitinase appears to belong to family 18, class III of chitinases. It can be detected in leaves or hydroponically-grown plants when exposed to As and also in soil-grown plants when EDTA is added as chelator and stimulator of metal mobility. Especially roots up-regulate the chitinase gene. This suggests a general defense mechanism of cells or tissue independent of photosynthetic pathways.

3.6 REFERENCES

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CHAPTER 4

DIFFERENTIAL EXPRESSION OF PROTEINS INDUCED BY LEAD IN THE DWARF SUNFLOWER *HELIANTHUS ANNUUS*

4.1 INTRODUCTION

Proteomic studies on plants have identified an array of proteins that are differentially expressed, many of which provide insights into the factors that mediate metabolic fluxes under normal and stressed conditions. For instance, with respect to metal toxicity, chitinases were expressed in the Dwarf Sunflower cultivar of *Helianthus annuus* in response to arsenic exposure (van Keulen et al., 2008), and in several other plants such as pea, bean, soybean, barley, and maize as the result of arsenic, Pb or Cd stress. In the latter case several isoforms of chitinases could be detected (Bekesiova et al., 2008). Other recent proteomic studies include induction of heat-shock protein 60 (Hsp60) in response to combined exposures of arsenic and heavy metals in *Platyonus pattulus* (Rios-Arana et al., 2005), proteins involved in sulfur metabolism in *Arabidopsis thaliana* roots by Cd (Roth et al., 2006) and induction of cysteine synthase in response to aluminum stress in rice (Yang et al., 2007).

This study aims to further expand some of the preliminary proteomic findings after exposure to heavy metals of the Dwarf Sunflower cv. Teddy Bear, a known hyperaccumulator. For this study, the controls were plants grown in clean soil and a stress condition of plants grown in soil contaminated with 30 mg/Kg each of Cd, Cr, and Ni. Cd, Cr, and Ni were selected as stressors to be representative of typical soil contamination levels in Northeast Ohio. Test conditions were when Pb was added as a contaminant.

4.2 MATERIALS AND METHODS

4.2.1. Reagents

The ZOOM immobilized pH Gradient (IPG) Runner system and electrophoresis and gel staining solutions were obtained from Invitrogen (Carlsbad, CA), whereas protein molecular weight markers were from Fermentas (Hanover, MD). Protein concentration was determined using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Other chemicals were obtained from Sigma–Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburg, PA).

4.2.2. Plant Materials and Metal Treatments

Seeds of the Teddy Bear (strain 06710K) cultivar of *H. annuus* were purchased from Jung Seed Company (Madison, WI). They were grown hydroponically using Rockwool, a

non-reactive, non-absorbent support system and sustained with a standard hydroponic nutrient solution (Cropking Corp., Seville, OH). After four weeks, the plants were transferred to a PVC trough and exposed to cadmium, nickel, and chromium (III), with and without lead at a complete recycle flow of 6 gallons per h. The metals were applied as Cd^{2+} ($\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$), Cr^{3+} ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$), Ni^{2+} ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$), and Pb^{2+} ($\text{Pb}(\text{NO}_3)_2$) at 40 IM each metal. One set of plants was subjected to a 4 l solution containing 40 μM of three metal ions (Cd, Ni, and Cr), the other containing Pb as well. Plants with no addition of metals were used as the control. After exposure, the plants were harvested and biomass was sectioned and weighed. The tissues were used for analysis of metal levels, and for protein and RNA isolation.

4.2.3. Protein Extraction for Two-Dimensional Gel Electrophoresis (2-DE)

This sample preparation procedure combined Trichloroacetic acid/acetone (TCA/acetone) precipitation (Jacobs et al., 2001) with SDS/phenol extraction (Wang et al., 2006) in order to reduce heavy streaks attributed to high levels of ribulose-1,5-bisphosphate carboxylase (RuBisCO) and background staining. Leaf material was ground in a mortar and pestle in the presence of liquid N_2 . Ground tissue powder (approximately 0.2 g per tube) was placed in 1.5– 2.0 ml capacity microcentrifuge tubes, suspended in 10% TCA/acetone with 0.07% β -mercaptoethanol (β -ME), mixed, and centrifuged for 3 min at 16,000g and 4⁰C. This step was repeated twice, followed by washing the pellet with ice-cold acetone containing 0.07% w/v β -ME twice. The pellets were air-dried, mixed with 0.4–0.8 ml (per 0.1 g plant material) of SDS buffer containing 30% sucrose, 2% SDS, 5% β -ME in 0.1 M

Tris-HCl, pH 8.0, Tris-buffered phenol, and vortexed thoroughly for 30 min at ambient temperature, centrifuged at 16,000g for 5 min at 4 °C. The upper phenol phase was then mixed with four volumes of 0.1 M NH₄OAc in MeOH:H₂O (4:1, v/v) and incubated overnight at -20 °C. It was centrifuged (14,000g, 5 min, 4 °C) and the pellet was then washed once with 0.1 M NH₄OAc in MeOH: H₂O (4:1, v/v) followed by an (500 µl) acetone:H₂O (4:1, v/v) washing. The pellet was air-dried and dissolved in 1% SDS buffer.

4.2.4. 2-D Gel Electrophoresis

For two-dimensional (2D) electrophoresis, proteins (200 µg) were first separated by isoelectric focusing (IEF) using the ZOOM IPG Runner system, pH 3–10 non-linear IPG strips and rehydration buffer (RB) containing 7 M urea, 2 M thiourea, 4% NP-40, 50 mM DTT, 1% ampholytes, and a trace of bromophenol blue according to the manufacturer's instructions. DTT (dithiothreitol) and bromophenol blue (3.2 µl/160 µl RB) were added into the buffer just prior to rehydration. The pellet obtained from phenol extraction followed by TCA/acetone precipitation was dissolved in rehydration buffer (160 µl). Samples were first applied in the mode of in-gel RB of the IPG strips and passive rehydration was carried out overnight at room temperature. IEF was performed at room temperature for 20 min at 200 V, 15 min at 450 V, 15 min at 750 V, and 1 h at 2000 V. Prior to the 2D analysis, the strips were equilibrated for 15 min in 10 ml equilibration solution (1 ml 10x sample reducing agent mixed with 9 ml 1x LDS sample buffer and subsequently for another 15 min in 10 ml of 125 mM alkylation solution (232 mg

iodoacetamide in 10 ml 1x LDS sample buffer). The IPG strips were transferred to NuPAGE Novex 4–12% Bis–Tris ZOOM gradient gel with IPG wells. Protein molecular weight marker was applied to the well provided on the gel for calibration of the molecular weight. The gels were run with an electrophoresis buffer (MES– SDS running buffer and electrophoresis was performed at 200 V for 35–40 min. Several protein stains were tested including Coomassie R-250 staining; however, Simply Blue Safe Stain (Invitrogen) was determined to be highly sensitive and gave better reproducibility in results. Therefore, all proteins spots analyzed were stained with Simply Blue Safe Stain. The protein spots were inspected visually and the most prominent differences in spot intensity were chosen for subsequent analysis.

4.2.5. In-gel Tryptic Digestion

Protein spots showing changes in abundance under combined exposure to four metals (Cd, Cr, Ni, and Pb) compared to three metals (Cd, Cr, and Ni) were manually excised for protein identification. The excised gels were sliced into approximately 1 mm³ pieces and washed/destained two times in CH₃CN–H₂O (200 µl 1:1, with 5% v/v AcOH) for 1 h at ambient temperature. The gel pieces were sequentially dehydrated in CH₃CN–H₂O (0.2 ml 1:1, with 5% v/v AcOH) for 5 min, rehydrated in 0.2 ml 100 mM (NH₄HCO₃) for 5 min, dehydrated in CH₃CN–H₂O (0.2 ml 1:1, with 5% v/v AcOH) for 5 min and dried in a SpeedVac. The samples were then reduced with 10 mM DTT and alkylated with 5 mM iodoacetamide prior to in-gel digestion. Samples were digested in-gel by adding 5 µl

promega gold sequencing grade trypsin (20 ng/ μ l) in 50 mM NH_4HCO_3 and incubated overnight at room temperature for complete digestion. Peptides were extracted from the gel with $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (2x 30 μ l, 1:1, v/v) containing 5% HCOOH . The extracts were combined and the volume was reduced to less than 10 μ l in a SpeedVac. Samples were then resuspended in 1% AcOH in a final volume of 30 μ l.

4.2.6. MS Analysis

ESI-MS/MS analyses were performed on a Finnigan LTQ linear ion trap mass spectrometer equipped with a microelectrospray ion source and an Eksigent automated nanoflow LC system. Reversed- phase separation of peptide digest was performed using fused silica capillary column (9 cm x 75 μ m i.d.) packed with Phenomenex Jupiter C18 (5 l). The peptide digest (10 μ l) was eluted from the column with a binary solvent gradient of $\text{CH}_3\text{CN}:\text{0.05 M AcOH in H}_2\text{O}$ at 0.3 μ l min^{-1} . The digests were analyzed using the data-dependent capability of the instrument, acquiring full-scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. The microelectrospray ion source was operated at 2.5 kV. The mass spectrometer was scanned over a mass (m/z) range of 400–1800 (1 microscan, 50 ms maximum ion accumulation time) of the most abundant peak with a collision energy setting of 35%. MS/MS spectra were obtained by collision-induced fragmentation of the most abundant peak in each primary scan, further analyzed to determine a peptide mass fingerprint.

4.2.7. Protein Identification

The digest was analyzed using the data-dependent multitask capability of the instrument. This mode of analysis produced approximately 2500 collisionally induced dissociation (CID) spectra of ions ranging in abundance over several orders of magnitude. Note that not all CID spectra were derived from peptides. The data were analyzed by using all CID spectra collected in the experiment to search the non-redundant NCBI (National Center for Biotechnology Information) database with an in-house MASCOT server with a green plant taxonomy filter. All matching spectra were verified by manual interpretation. The peptides that were not matched in the NCBI database search were manually interpreted and the resulting amino acid sequence was used to search the NCBI database with the FASTA program for homologous protein sequences. The interpretation process was aided by additional searches using the programs SEQUEST and BLAST as needed. After initial identification of peptides in the NCBI database and identification of possible candidate proteins, the Sunflower Gene Index was searched for these proteins as present in temporary consensus (TC) sequences and putative candidates scrutinized for peptide matches.

4.2.8. RNA Isolation and Analysis

RNA was isolated and subjected to RT-PCR as described previously (van Keulen et al., 2008). Primers were designed based on the DNA sequence of thaumatin, TC 40600, with sequences: sense: 5' ATGACTTGTGCCAAAACCTTCTACTC and anti-sense: 5' ATATACTTAAAGAGTTTATGGACAGAAC, which will amplify a 705 basepair (bp)

DNA fragment after reverse transcription of the RNA with the anti-sense primer. The PCR was done using 10% of the cDNA reaction, as described (van Keulen et al., 2008) and the DNA was analyzed on a 1% agarose gel and stained with ethidium bromide. As control on RNA input a 400 bp fragment of large subunit rRNA was amplified using rDNA specific primers and subjected to the same method.

4.3. RESULTS AND DISCUSSION

The Dwarf Sunflower is capable of hyperaccumulating toxic metals. The aim of this study was to provide a preliminary screen to identify proteins that are expressed in leaves of *H. annuus* under stress by adding Pb to the three normally found metals (Ni, Cd, and Cr) in the industry-polluted soils of Northeast Ohio. As an initial screen, SDS-PAGE was performed on both Sundance and Teddy Bear cultivars. The gel electrophoresis pattern is shown in Figure 18.

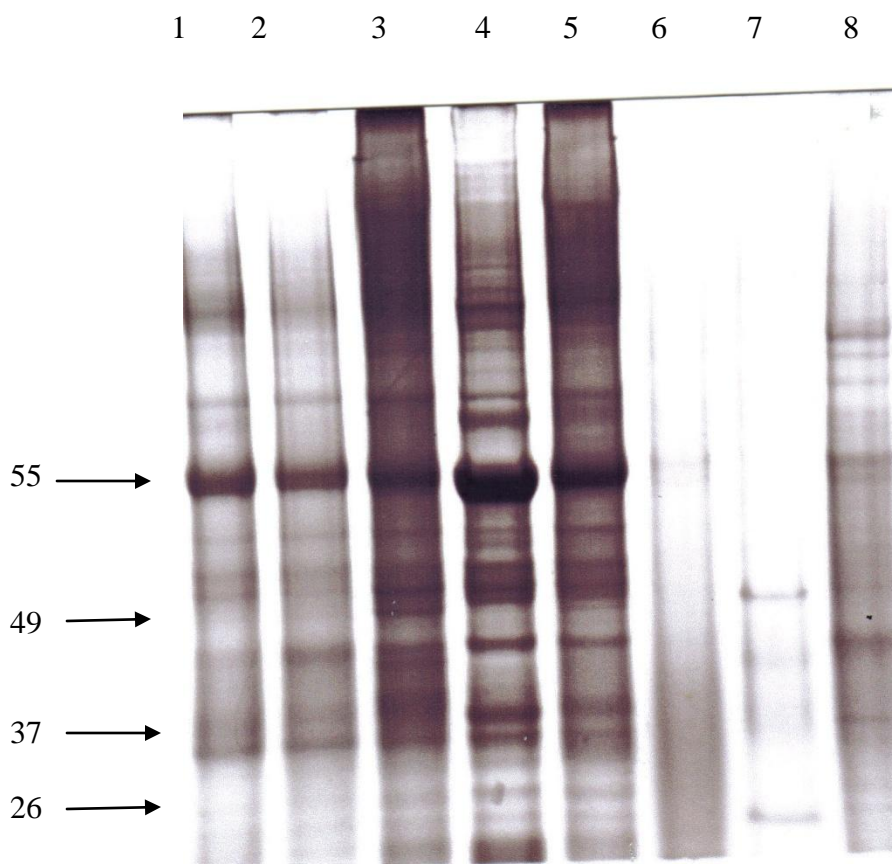


Figure 18: One dimensional polyacrylamide gel electrophoresis of Sunflower proteins.

Fifty μg protein isolated from leaves was loaded per lane except lanes 6 - 8 which contained about 10 μg of protein isolated from stems. Lane 1: SD control, lane 2: SD (As), lane 3: SD all metals, lane 4: TB control, lane 5: TB all metals, lane 6: TB stem control, lane 7: TB stem all metals, lane 8: SD stem control. SD: Sundance, TB: Teddy Bear, all metals is Ni, Cd, Cr + Pb. As: arsenate only. The gel was stained with Simply Blue Safe Stain. Three bands were excised from lane 3 and one each from lanes 5, 7, and 9 (SD stem all metals, not shown in figure).

Six bands could be identified to be unique in samples exposed to lead, which were excised and submitted for mass spectrometry analysis. Because the separation was on a one-dimensional gel more than one protein can be expected in each band. However, similar bands in different samples gave the same results. Selected were those proteins that had the highest percentage sequence covering of the protein and at least three individual peptides for each protein. The results are summarized in Table VII.

Table VII: Lead-induced proteins

Band number	Source	Mr (gel)	Identity(ms Mr)	% coverage
1	SD leaves	49 kDa	ATP synthase β (50)	18
			ATP synthase α (51)	20
2	SD Leaves	37 kDa	Malate Dehydrogenase (MDH)	18
			ATP synthase α and β	20, 16
			Carbonic anhydrase (36)	23
3	SD leaves	32 kDa	Triosephosphate isomerase (20)	48
			MDH (37)	16
			Light harvesting protein (28)	16
			RubisCO activase (30)	19
4	TB leaves	49 kDa	4-nitrophenylphosphatase (33) ^a	20
			MDH	12
5	TB leaves	49 kDa	MDH	12
6	SD stems	49 kDa	MDH	22
			GAPDH (37)	20

^a. 4-nitrophenylphosphatase belongs to a large family of proteins known as haloacid dehalogenases (HAD), one of these is found in plants and is 2-phosphoglycolate phosphatase.

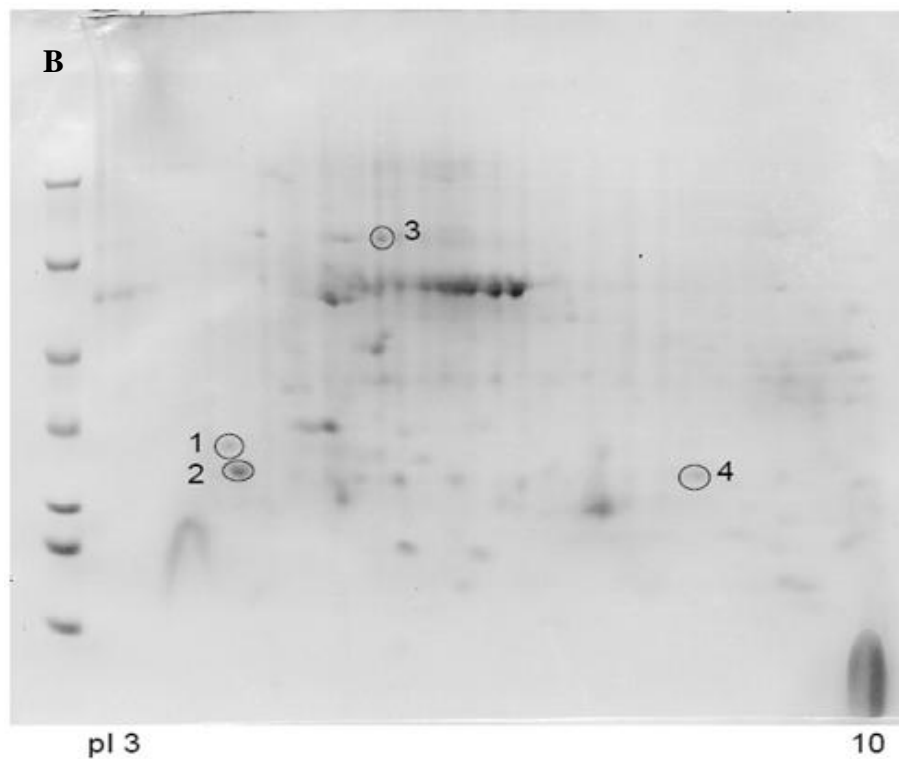
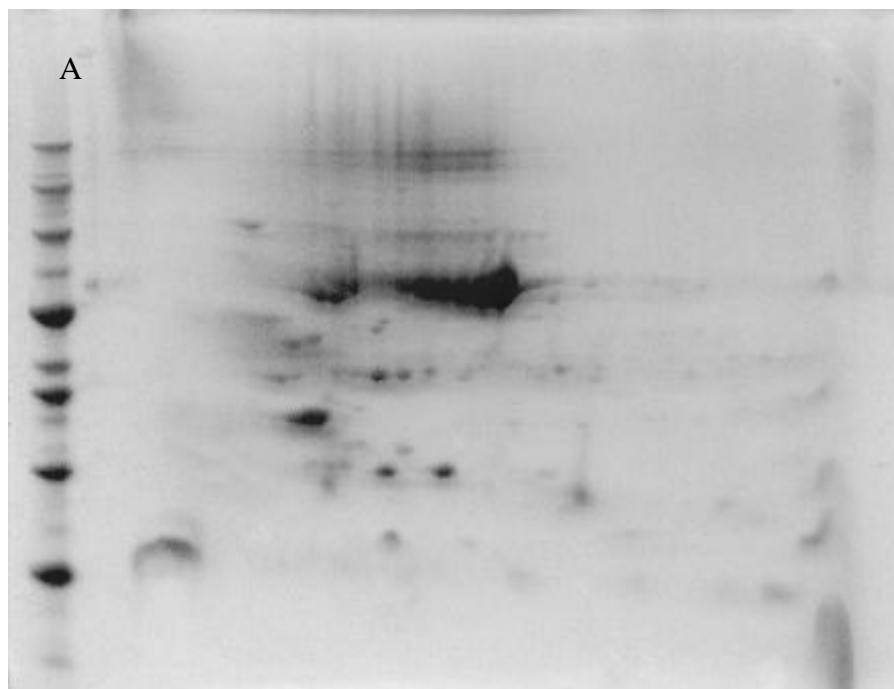


Figure 19: 2-D gel of leaf proteins obtained from Teddy Bear cultivars

2-DE separation of soluble leaf proteins. Leaf proteins were isolated from the cultivar Teddy Bear and 200 μ g subjected to 2-DE separation and stained with Simply Blue Safe Stain. **A:** Plants were exposed to Cd, Cr and Ni only and **B** plants were exposed to Cd, Cr, and Ni plus Pb. The molecular mass of the markers is in kDa ranged from 14 to 116.

By using two-dimensional gel electrophoresis four proteins could be identified that were clearly over-expressed in the presence of all four metals, but were absent or undetectable when Pb was omitted (Fig. 19). In order to ensure reproducibility of gel patterns, a minimum of three gels was used for each condition. These protein spots were not visible in the control conditions with no metal added or under the stress conditions by three metals (Ni, Cd, and Cr), omitting the Pb (Fig. 19A). The areas where these three spots appeared were empty of proteins in gels containing proteins from leaves derived from untreated (not shown) or Ni, Cd, and Cr treated plants. This suggested that these proteins were strongly induced under Pb toxicity. Whether these proteins would change in abundance with time or depended on metal concentration was not examined.

Two protein spots (spot 1 and 2) corresponded to the marker region with molecular mass of 25–35 kDa and pI value between 4 and 5. Another spot (spot 3) was around the marker region of 80–90 kDa and pI of 5–7. The fourth spot (spot 4) was around the marker region at 25–35 kDa with pI value of 8–10. It should be pointed out that *H. annuus* contains high levels of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a 53 kDa protein, as was indicated by heavy streaks on 2D gels, masking a large number of proteins in this molecular mass region and over a wide pI range. Therefore, attempts were made to reduce it during sample preparation. In the process, various proteins including those differentially expressed might also have been either removed or reduced. MS/MS analyses indicated a complex mixture of proteins identified by both single and multiple peptide database matches to MS/MS spectra. All proteins identified in the selected spots are listed in Table VIII.

Table VIII: Peptides aligned with known database sequence.

[M+H] ⁺	Sequenced peptide ^a	NCBI database ^b	% sequence coverage
Spot 1: chitinase			21
92	NQLLPAIR	NQLLPAIR	
977	VFLSIGGQR	VFLSIGGQR	
1113	YGGVM _o LWDR	YGGVMLWDR	
1522	FYDKQSGYSDAIK	FYDKQSGYSDAIK	
1864	VYLSAAPQCPFPSGDVR	VYLSAAPQCPFPSGDVR	
1876	ACQAQNVKVFLSIGGQR	ACQAQNVKVFLSIGGQR	
1992	KVYLSAAPQCPFPSGDVR	KVYLSAAPQCPFPSGDVR	
Spot 1: chloroplast drought-induced stress protein CDSP-34			15
1122	SDGGSVVFVXXK	GDAGSVFVLLK	
1300	QXVDSM _o YGTNR	QLADSLYGTNR	
1443	AEVVEXXTQXEAK	AEIVELITQLESK	
1504	GXXTSVQDTASNVAK	GLLTSVQDTASSVAK	
Spot 2: thaumatin-like protein			43
Sunflower Gene Index^c			
621	VVFCP	VVFCP	
1499	TQGGCINNPTVYK	TQGGCINNPTVYK	
1604	CAGNLNCEPKDXR	CAGNLNCEPNELR	
1640	PXECP	ECP	
2027	GTVGEVSWQXTVAPGTAQAR	LENGQSWQITVAPGTAQAR	
2184	RGTVGEVSWQXTVAPGTAQAR	RLENGQSWQITVAPGTAQAR	
2228	TNEYCCTNGPGSCGPTPXS	TNEYCCTNGPGSCGPTPLSR	
854	CPDAYSYQDDPTSXF	CPDAYSYQDDPTSLFTCP	
	GGTNYK	GGTNYK	
2983	DVXXECQ	DVINQCQY	
Spot 3: heat-shock cognate 70-1			29
Sunflower Gene Index			
1082	XXQDFFNGK	LLQDFFNGK	
1165	XQVEYKGEAK	IQVYEGERAR	
1198	FEXTGXPPAPR	FELSGIPPAPR	
1214	EAGTXAGXNVXR	DAGVISGLNVMR	
1228	VEXXANDQGNR	VEIANDQGNR	
1254	FEEXNADXFR	FEELNMDLFR	
1257	MoKETAEAYXGK	MKEIAEAFLGT	
1268	MoVNHFVSEFK	VNHFVQEFK	
1344	EXEAXCNPXXTK	ELENVCNPIIAK _d	
1482	ARFEEXNADXFR	ARFEELNMDLFR	
1488	TTPSYVAFTDTER	TTPSYVAFTDSER	
1600	XXNEPTAAAXAYGXDK	LINEPTAAAIAYGLDK	
1694	NQVAMNPTNTVFDAK	NQVAM _o NPNTXFDAK	
1788	XXNEPTAAAXAYGXDKK	LINEPTAAAIAYGLDKK	
1993	AXKNAVVTVPAYFNDSQR	TVKNAVVTVPAYFNDSQR	
2112	SXNPDEAVAYGAAVQAAXXHGDK	SINPDEAVAYGAAVQAAILSGE	

[M+H] ⁺ coverage	Sequenced peptide ^a	NCBI database ^b	% sequence
Spot 3: ribulose-1,5-bisphosphate carboxylase/oxygenase			
		NCBI database	18
737	GGXDFTK	GGLDFTK	
733	DDFXEK	DDFIEK	
857	ACYECXR	ACYECLR	
1022	DTDXXAAFR	DTDILAAFR	
1060	VAXEACVQAR	VALEACVQAR	
1408	XTYYPEYETK	LTYYPEYETK	
1446	YGRPXXGCTXKPK	YGRPLLGCTIKPK	
1468	DDENVNSQPFMoR	DDENVNSQPFMR	

^a All sequenced peptides: X = I or L, Mo = oxidized M.

^b From MASCOT screen of the NCBI database.

^c From secondary screen against the Sunflower Gene Index.

^d From a different/overlapping HSP70 sequence.

Two proteins identified in spot 1 matched to chitinase, a type III chitinase, EC 3.2.1.14, of *H. annuus* with a Mr of 32 kDa and a chloroplast drought-induced stress protein (CDSP-34) of *Solanum tuberosum* with Mr of 36 kDa (Langenkamper et al., 2001), which was identified by four peptides covering 15% of the protein sequence. There was no match in the NCBI protein database for this protein or family of any *Helianthus* species. It appeared to be a minor component of the spot based on the overall lower abundance of the peptides compared to the chitinase-derived peptides.

Chitinase is an abundant protein expressed in the presence of all four metals and is clearly up-regulated when Pb is present. As shown in the previous chapter it was demonstrated that As was also capable of inducing this chitinase in the cultivar Sundance. However in the presence of Cd, Cr and Ni a low level of chitinase could be detected. The same combination of metals (Cd, Cr, or Ni) or each individual was unable to up-regulate chitinase in the Teddy Bear cultivar, see chapter 3, figure 16. This response is different as compared to the response to Cd in pea plants. In this case chitinase was up-regulated when

plants were exposed to Cd (Rodriguez-Serrano et al., 2009). Apparently different plants, and even cultivars of one species respond in a different way to metal stress since pea, bean, soybean, barley, and maize up-regulated a variety of chitinases as the result of As, Pb, or Cd stress (Bekesiova et al., 2008).

In the Dwarf Sunflower, both As and Pb may trigger a common defense mechanisms at the transcriptional level (van Keulen et al., 2008) of which chitinase is the easiest to identify. Other studies have also shown that chitinases were expressed in a broad range of stressful conditions ranging from fungal and bacterial infection to abiotic stresses such as light and temperature change (De Los Reyes et al., 2001). They belong to the systemic acquired resistance and pathogenesis-related (PR) proteins (Heitefuss, 2001; van Loon et al., 2006). Chitinase appears to counteract oxidative stress as was shown by Dana et al. (2006) using transgenic tobacco plants that over-expressed a fungal chitinase. The plants were not only more resistant to fungal infection but also to salt and metal ion stress.

Chloroplast drought-induced stress protein (CDSP) is a member of the plastid associated protein (PAP) fibrillin family. This protein was shown to be expressed in a variety of plants under such stresses as drought, high light exposure, oxidizing environment, bacterial infection, and mechanical wounding (Langenkamper et al., 2001; Chen et al., 1998; Jones et al., 2006). Although the Sunflower Gene Index (The Gene Index Project of the Dana Farber Cancer Institute, <http://compbio.dfci.harvard.edu/index.html>) contains PAP-fibrillin-like protein sequences, the ones in the database do not match the peptides observed.

The peptides in spot 2 matched to a thaumatin-like protein of *S. tuberosum*, a 25 kDa protein. It was identified by six peptides covering 43% of the protein's sequence. A search of the Sunflower Gene Index indicated a thaumatin-like protein (TC 40600) with an even higher

degree of identity in the peptide sequences (see Table VII). Only four amino acids differ as can be expected since the peptides were derived from a Dwarf Sunflower. Thaumatin-like proteins are functionally similar to chitinases in that they are expressed in plants in response to fungal and viral stresses, as well as abiotic stress factors such as increased salinity and drought (Selitrennikoff, 2001; Brandazza et al., 2004; Menu- Bouaouiche et al., 2003). Thaumatin-like proteins are also induced as the result of H₂O₂ formation in transgenic sunflowers (Hu et al., 2003). Table IX is representative of coverage of the peptides observed within the entire protein sequence of thaumatin, identified in spot 2.

Table IX: Sequence of a Sunflower Thaumatin-like Protein.

mtcaknllsiltllsigcftltqgatl**DVINQCQY**pvwaawasttpgggkrlengq**SWQITVAPGTAQAR**iwgtrtgcnfndan
 grgrcdtgdcmgmlccqgygappntlaefalnqdnndfvdslvdgfnipmefspvgascktmr**CAGNLNGECPNELRTQ**
GGCNPCTVYKTNEYCTNGPGSCGPTPLSRFfkdr**CPDAYSYPQDDP**SLFTCPGGTNYK
 VVFCP

The matching peptides are indicated in capital letters. The four amino acids in italics and red are mismatches between the peptides from the Dwarf Sunflower sequence and the “normal” sunflower in the Sunflower Gene Index.

To show that the thaumatin gene was transcriptionally activated as has been shown for the chitinase gene (van Keulen et al., 2008), reverse transcription followed by the polymerase chain reaction (RT-PCR) was performed on RNA purified from leaves derived from control plants and plants exposed to arsenic or lead using thaumatin specific primers. As loading control for RNA, the same amounts of RNA from the same samples were used for RT-PCR using primers specific for part of the large subunit rRNA. The results, shown in Fig.

20, indicate that the thaumatin gene was not expressed in control plants but transcription was up-regulated in As- and even much stronger in Pb-treated plants. The DNA resulting for RT-PCR was subjected to DNA sequence analysis and was shown to be thaumatin specific (not shown). Simultaneous expression of chitinase and thaumatin, a PR-5 type protein (van Loon et al., 2006) is typical for sunflower, since expression of this protein was not correlated with chitinase expression in Tobacco plants (Dana et al., 2006).

The peptides identified in spot 3 matched to HSP70 of *A. thaliana*. Hsp70 was identified by 16 peptides covering 29% of the protein sequence. A search of the Sunflower Gene Index indicated a partial sequence for Hsp70 (TC 39983) with only a few differences in amino acids (see Table VIII). Another protein in spot 3 was identified as a large subunit of RuBisCO of *H. annuus*. It was identified by nine peptides covering 18% of the protein sequence. The peptides given in Table VIII compare the observed amino acid sequences to the database sequences. The heat-shock proteins are also commonly induced under stressful conditions. Studies have shown that members of the Hsp70 family were up-regulated as a result of thermal and oxidative stress including heavy metal exposure (Wang et al., 2004). They play a broad range of roles including protein folding, assembly, translocation, and degradation. In many normal cellular processes, stabilization of proteins and membranes, and can assist in protein refolding (Wang et al., 2004). Further, the Hsp70 proteins act as a molecular chaperone by binding to hydrophobic residues of protein denatured under stress conditions, preventing proteins from random aggregation, and helping to refold to their native conformations (Wang et al., 2006). They also assist in the transport of various proteins across membranes by inducing changes in their structural conformation (Wegele et al., 2004). It is intriguing to note that the heat-shock response is controlled by heat-shock

transcription factors that respond to oxidative stress and could act as possible H₂O₂ sensors (Miller and Mittler, 2006).

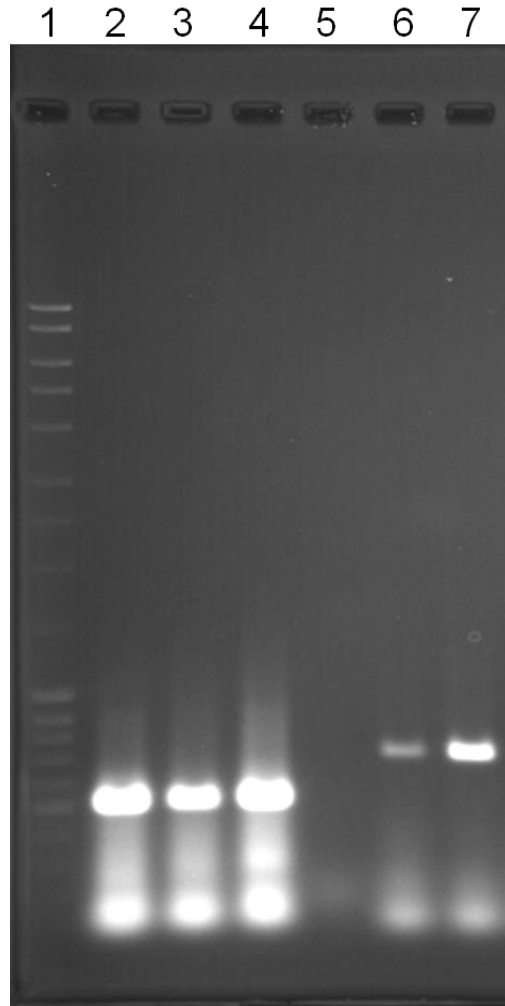


Figure 20: RT-PCR of total RNA samples.

Reverse transcription was performed on 5 µg total RNA isolated from leaves as indicated using specific anti-sense primers. Of each reaction, 10% of the cDNA product was subjected to PCR using 30 cycles with gene specific primers. Each reaction was size fractionated on a 1% agarose gel and stained with ethidium bromide. Lanes 2–4, control reactions using rRNA specific primers. Lanes 5–7, reactions using thaumatin specific primers. Lane 1: DNA size marker, the 0.5 kbp and 1 kbp markers are indicated, the DNAs between the markers differ in 100 bp. Lanes 2 and 5: RT-PCR using RNA isolated from control leaf samples, lanes 3 and 6, RNA from As treated samples and lanes 4 and 7, RNA from Pb-treated samples

The large subunit of RuBisCO was also identified in spot 3. This protein is so abundant that it is a general contaminant in various spots both in 1-D and 2-D gels. In addition it appears in lower molecular weight areas, indicating degradation, as was already shown in Chapter 2.

The differentially expressed proteins were thus a class III chitinase, a thaumatin-like protein, possibly a chloroplastic drought-induced stress protein (CDSP), and heat-shock protein 70. Expression of these proteins observed in *H. annuus* suggested that several genes in *H. annuus*, as in many other plants, are regulated by abiotic stresses and that there may exist a central surveillance mechanism that elicits common stress responses. For example, genes that are regulated by pathogens such as genes encoding chitinase also seem to respond to the presence of toxic metals. The induction of chitinase and thaumatin suggests that a common stress-induced pathway is operating after poisoning with Pd or As similar to pathogen exposure or peroxide exposure. The stress-related proteins expressed in this study are not known as metal ligands, and therefore do not form complexes with metals. Their expression does not imply their role in metal detoxification but can be seen as a general response to stress using a similar pathway as that required after infection with pathogens, drought, peroxides, and probably other reactive oxygen species (ROS), with other words they are defense-related proteins. As and Pb appear to have the same toxic effects on the sunflower, although they behave differently, As being a phosphate analog and Pb forming a phosphate–Pb complex. Further analysis will be needed to establish why chitinase and thaumatin are expressed in the presence of As and Pb.

Peptides in spot 4 could not be matched to any sequence in the NCBI database or the Sunflower Gene Index.

Another observation is that metal poisoning results in damage to proteins and possibly damage to the photosynthetic structures. As shown in chapter 2, the amount of RuBisCO diminishes when proteins are analyzed by standard protein gels. When mass spectroscopy was used on proteins purified from both 1-D and 2-D gels, many bands and spots showed the presence of the large subunit of RuBisCO, apparently as minor contaminants of many of the lower molecular weight proteins. So it is clear that the enzyme is subject to degradation. This has been observed by others as well (Gillet et al., 2006).

In the 1-D gel analysis by mass spectrometry several proteins could be identified and their presence was consistently observed in several samples in the same sized protein bands and showed a large coverage of the sequence. Thus their identity seems plausible. The enzymes and proteins detected are all part of the photosynthetic system and are up-regulated as the result of damage to this system. The degradation of RuBisCO must have a major effect on metabolism. With other words, the up-regulated proteins appear to be present to compensate for photosynthetic loss. One of the proteins is a light harvesting chlorophyll a/b binding protein (LHCP) and might be up-regulated to increase light harvesting potential of damaged thylakoid membranes.

Most interesting is the up-regulation of RuBisCO activase, which is needed for activity of RuBisCO and has been called RuBisCO's molecular chaperone (Carmo-Silva and Salvucci, 2011). The activase catalyses the binding of CO₂ to form a carbamate in RuBisCO, which allows the enzyme to become catalytically competent for its substrates ribulose 1,5 biphosphate, CO₂ and O₂. Thus the up-regulation of the activase might be to compensate for the loss of active RuBisCO. In addition up-regulation of carbonic anhydrase (CA) and phosphoglycolate phosphatase (PGP) are consistent with this observation. CA catalyzes the

formation of CO₂ gas from the soluble bicarbonate form and so increases the concentration of accessible CO₂ for RuBisCO. RuBisCO also catalyzes the photorespiration of ribulose 1,5 biphosphate to 3-phosphoglycerate and 2-phosphoglycolate. The 2-phosphoglycolate would be a loss to photosynthesis, it cannot enter the Calvin cycle or can be converted to carbohydrates, but is recovered through the action of PGP to become glycolate, which is converted to glyoxalate and further used in a recovery pathway that will recover $\frac{3}{4}$ of the carbons (Reumann and Weber, 2006; Schwarte and Bauwe, 2007).

The increase of MDH has been reported by others (Shao et. al., 20011; Bona et al., 2010) as an indication of damage and its resulting change in respiration. The fact that MDH was found both in leaves and stems validates this result. MDH is one of the enzymes involved in the regeneration of glyoxylate. The RuBisCO activase requires ATP, this is probably also the reason that ATP synthase subunits were found to be up-regulated. The up-regulation of GAPDH will be discussed further in the next chapter.

4.4 CONCLUSION

Proteomic analysis of leaves from the Teddy Bear cultivar of *H. annuus*, indicated differential expression of many proteins, four of these were abundantly over-expressed and easily detectable when the plants were subjected to stress by a mixture of Cd, Ni, Cr, and Pb and the use of 2-D gels. The proteins were not visible in the control samples without metal stress or when Pb was omitted from the metal solution, suggesting that toxicity mechanisms of Pb is different from the other three metals tested. Analysis of the spots by LC-MS/MS showed up-regulation of chitinase, chloroplastic drought-induced stress protein (CDSP-34), a thaumatin-like protein, and heat-shock cognate 70, which points to the activation of a general defense mechanism. In addition MDH, GAPDH and ATP synthase appear to be up-regulated as well, which points to the toxic effect of Pb on cellular respiration. The coinciding activation of carbonic anhydrase, RuBisCO activase and 2-phosphoglycolate phosphatase suggests compensation of the loss of RuBisCO and salvage of the remaining enzyme activity and resulting products.

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CHAPTER 5

DIFFERENTIAL PROTEOME ANALYSIS OF *CHLAMYDOMONAS REINHARDTII* RESPONSE TO ARSENIC EXPOSURE

5.1 INTRODUCTION

Previous investigations using proteomic methods combined with mass spectrometry revealed that the Dwarf Sunflower, *Helianthus annuus*, is able to accumulate As and this accumulation results in the expression of stress-related proteins such as chitinase, as is described in chapters 3 and 4. It can be assumed that the physiological and biochemical response towards As stress is highly complex since higher plants are organized as multicellular organisms with many types of tissues, which results in a complex proteome and stress-related responses that are at the cellular, tissue and organismal levels. Moreover, mass spectrometry relies heavily on the availability of a genomic database for the organism or cells under investigation, which is not available for certain plants such as *H. annuus*. Therefore, it was decided to investigate whether a single cell organism could be used to study stress response at the cellular level without the impediment of complex organ systems and one for which a genome database is available.

In this study, the unicellular green alga *Chlamydomonas reinhardtii* was chosen in order to perform proteomic analysis to analyze its response to arsenic. The alga's complete genome is available (Merchant et al., 2007) and research on its proteome and metabolome are being performed (May et al., 2008) and its sub-proteome (Wagner et al., 2009) and chloroplast proteome have been studied in detail (Terashima et al., 2011). It is easy to grow, has a short-life cycle and a large number of experimental parameters can be incorporated into the study. In addition, data so far obtained for *C. reinhardtii* are similar to those obtained for higher plants, suggesting that it might be a good model organism. For example, recent work showed that phytochelatins (PC) are the major chelators induced upon cadmium (Cd) treatment and that these complexes sequestered up to 70% of the total Cd found in Cd-treated cells (Collard et al., 1990, Hu et al., 2001). Other studies on *C. reinhardtii* discussed the fact that it might be a good model to study heavy metal homeostasis and tolerance (Hanikenne et al., 2003), light stress (Förster et al., 2006) transition metal transport (Rosakis et al., 2004), and the role of PCs in Cd accumulation (Nishikawa et al., 2006). A proteomics study on the effect of Cd showed down-regulation of a number of proteins involved in photosynthesis, Calvin cycle and Chlorophyll biosynthesis, at the same time some typical stress-related proteins were up-regulated such as heat shock proteins, superoxide dismutase and glutathione-S-transferase to name a few of the proteins identified (Gillet et al., 2006).

However, very little is known about arsenic toxicity in *C. reinhardtii*. Many have reported on arsenic toxicity and have described effects such as anti-oxidative response (Srivastava et al., 2005), changes in uptake kinetics and arsenic-phosphate interactions (Wang et al., 2002) mainly using *P. vittata*. Some studies using arsenic treatment have been reported using *H. annuus* (Raab et al., 2005) as well as *C. reinhardtii* (Kaise et al., 2005,

Kobayashi et al., 2005). But other than a few proteomic-level studies about arsenic accumulation such as in rice plants (Ahsan et al., 2010) and in the Dwarf Sunflower (van Keulen et al., 2008) and *Pteris vittata* (Bona et al., 2010), very little has been reported about proteome changes upon arsenic stress in *Chlamydomonas*. This study is based on the premise that *C. reinhardtii* might be useful to investigate the effects of As exposure and to search for differential expression of proteins in order to see 1) if the response also includes oxygen stress and metabolic stress as found in the Dwarf Sunflower 2) if a general defense mechanism is present and 3) if other proteins such as metal-binding proteins could be identified.

5.2 MATERIALS AND METHODS

5.2.1 Cell Growth and Arsenic Treatment

C. reinhardtii wild type (+) was purchased from Carolina Biological Supply Co. (<http://www.Carolina.com>) and algae were purified from contaminating bacteria by serial dilution and plating out of cells on agar plates. The purified cells were grown at 25 °C in 250 ml foam-plugged (Jaece Industries, <http://www.jaece.com>), autoclaved Erlenmeyer flasks containing 100 ml growth medium. The growth medium was composed of Tris-acetate-phosphate (TAP) as described by Dunford et al. (<http://www.unbf.ca/vip/restools/TAP.htm>). The flasks were shaken continuously (130 rpm) on an orbital shaker incubator using continuous illumination using 15 W "Plant & Aquarium" fluorescent lighting. Experiments were conducted to establish the toxicity ranges using dibasic sodium arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$). The concentration of arsenate used was 0, 100, 200, 300 and 400 μM . The flasks were

inoculated with 1×10^5 cells ml^{-1} . Cells were counted with a Levy-Hausser hemacytometer. Cell growth was monitored at least once a day until the experiment was terminated at the end of a six day period. The growth rate was determined by counting live cells in a hemacytometer and optical density at 750 nm. Cells were harvested when the density reached 0.6 - 0.7 optical density units for each concentration of arsenate. Each series of 0 – 400 μM arsenate was done twice, the first time in triplicate, the second time in duplicate. For the growth rate only live cells were counted. For proteomics the cells were collected by centrifugation at 6000 x g for 20 min at 20 °C. The cell pellets were stored at –80 °C or immediately processed for extraction of total proteins.

5.2.2 Sample Preparation for Gel Electrophoresis

After six days of incubation cells were harvested and pellets were ground to a fine powder in liquid N_2 . Approximately 1g of ground material was resuspended in 1 ml of 1x phosphate buffered saline (PBS) at pH 7.4 containing 1x nuclease solution (100 mM Tris-HCl, pH 7.0, 50 mM MgCl_2 , RNase (500 $\mu\text{g}/\text{ml}$) and DNase (1mg/ml)) and 1x plant protease inhibitor solution (G-Biosciences, <http://www.gbiosciences.com>). The suspension was vortexed briefly and 3 freeze-thaw cycles were performed in order to break the cell walls. The suspension was sonicated on ice six times for 10 s each with intermittent 1 min breaks using an ultrasonicator (Branson Ultrasonics, <http://www.All-Spec.com/BransonUltrasonics>). Samples were centrifuged in 3 cycles successively at 4 °C with the settings of 15,000 x g for 30 min; 20,000 x g for 30 min; and 20,000 x g for 15 min in order to obtain a clear protein extract without any debris. Protein precipitation was performed using

freshly prepared ice-cold, saturated ammonium sulfate ((NH₄)₂SO₄) solution. The first protein precipitation was performed by adding saturated ammonium sulfate drop-wise until the final concentration reached 40%. After 30 min incubation in an ice bath, the suspension was centrifuged at 15,000 x g for 20 min at 4 °C. The supernatant was subjected to a second precipitation by increasing the salt concentration up to 60% ammonium sulfate and a clean protein pellet was obtained. The protein pellets were resuspended in 1 x PBS buffer and dialyzed overnight at 4 °C against 1 x PBS buffer. Protein concentration in each fraction was determined using the Bradford assay. Five hundred micrograms (500 µg) of protein from the 60% ammonium sulfate fraction was precipitated with 10% Trichloroacetic acid (TCA)/acetone with 0.07% dithiothreitol (DTT) at -20 °C overnight. After centrifugation (17,000 x g for 20 min at 4 °C), the protein pellet was washed twice with ice-cold acetone, centrifuged (17,000 x g for 20 min at 4 °C), vacuum dried and stored at -80 °C until use.

5.2.3 Two-Dimensional Protein Gel Electrophoresis

Protein pellets were resuspended in freshly prepared rehydration buffer (RB) (7M Urea, 2M thiourea, 1% CHAPS, 1% Triton X-100, 1% DTT and 0.2% Ampholytes and 1% BromoPhenolBlue). A total of 500 µg of protein was used for 2-DE analysis. Each pellet was resuspended in 200 µl of rehydration buffer at room temperature. Rehydration was performed by loading the total 200 µl RB on 11 cm pH 3-10 linear immobilized pH gradient (IPG) strips (BioRad, <http://www.discover-bio-rad.com>) into the tray. Active rehydration was performed at 5V for 14-16 h at room temperature. The IPG strips were focused using a Protean IEF cell (Bio-Rad) according to the manufacturer's instructions and the total volt-hours reached at the

end of IEF was 40 kVh. After isoelectric focusing was completed, proteins were separated using a 12% SDS-PAGE gel (Bio-Rad) at a voltage of 200 V. The gels were fixed in a 100 ml fixing solution (50 % ethanol, 10% acetic acid in water) for 30 min at room temperature with gentle shaking. After washing with water, the gels were stained overnight with GelCode Blue staining solution (Pierce, <http://www.piercenet.com>).

5.2.4 Image Analysis of Two-Dimensional Gels

Gels were scanned at 300 ppi on a Scan maker, 5900 scanner, (MicroTek Lab Inc, <http://www.microtek.com>) and the scanned images of protein spots were loaded into the PDQUEST gel analysis software (Version 6.2, BioRad). The data set was obtained from gels using proteins from control and arsenic-treated cells. The proper spot detection parameters were selected and adjusted using the Spot Detection Wizard in order to identify and count all the protein spots of interest in the gels. Match sets were created using all gels and up-regulated/over-expressed spots at different concentrations of arsenic exposure were determined using a Student's t-test within PDQUEST with a significance level set to 95% ($p < 0.05$). Replicates from three independent extracts were used for analysis. Protein spots were finally visually inspected to validate the obtained results.

5.2.5 In-gel Tryptic Digestion

Stained protein spots that appeared as up-regulated after As treatment were selected and excised manually. They were cut into smaller pieces and transferred to 1.5 ml microcentrifuge tubes. The excised gel fragments were washed twice for 1 h each with 175 μ l

of wash reagent (50 % ethanol (95% USP grade), 5% acetic acid (99.9% Aldrich, <http://www.sigmaaldrich.com>) and water) to remove the dye that was bound to the protein. It was then dehydrated for 5 min using 175 μ l of HPLC grade acetonitrile (Burdick and Jackson, <http://www51.honeywell.com>) and rehydrated for 5 min with 100 mM ammonium bicarbonate. It was again dehydrated for 5 min with acetonitrile and vacuum-dried in a SpeedVac for approximately 3 min. After this dehydration/rehydration procedure, the gel pieces were digested with trypsin by adding 10-15 μ l of 10 ng/ μ l trypsin reagent (Promega sequencing grade modified trypsin dissolved in 2 ml 50 mM ammonium bicarbonate, <http://www.promega.com>). The gel pieces were vortexed, centrifuged briefly and digested overnight at room temperature. The resulting peptides were extracted from the polyacrylamide in two aliquots of 30 μ l of extraction reagent (50% acetonitrile, 5% ACS grade formic acid). These extracts were combined and evaporated to less than 10 μ l in a SpeedVac and then resuspended in 1% acetic acid to make a final volume of approximately 30 μ l for LC-MS/MS analysis.

5.2.6. Mass Spectrometry Analysis and Protein Identification

The mass spectrometric analysis for the identified protein spots from the treated gels were performed using either LCQ-Deca or LTQ Linear ion trap mass spectrometer (ThermoScientific, <http://www.thermoscientific.com>) located in the Proteomics Core, Lerner Research Institute, the Cleveland Clinic, Cleveland, OH. The HPLC column was a self-packed 10 cm x 75 μ m Phenomenex Jupiter C18 reversed-phase capillary chromatography column. One to ten μ l of the tryptic digests were injected and the peptides were eluted from

the column by an acetonitrile / 0.05 M acetic acid gradient at a flow rate of 0.2 μ l/min. The nanoelectrospray source was operated at 2.5 kV and the mass spectrometer was operated in a data-dependent mode.

The *Chlamydomonas* protein database was obtained from the *Chlamydomonas* center JGI portal chlre4 genome browser at <http://genome.jgi-psf.org/Chlre4/Chlre4.home.html>. It was uploaded into the MASCOT search program in order to identify the proteins analyzed from the mass spectrometer. The LC-MS/MS data (from Xcalibur raw files) for each sample was searched against the above mentioned *Chlamydomonas* protein sequence database and additional searches were performed using the full NCBI non-redundant database (The Genetic sequence database at the National Center for Biotechnology Information). Protein versus protein alignment searches were performed for each protein sequence as well as for the peptide sequences obtained. The identity of these proteins was further proved by SEQUEST, tandem mass spectrometry database searching algorithm on Bioworks software (Thermo Finnigan, <http://www.sisweb.com>). All the MS/MS spectra were searched against the databases generated from identified protein sequences for each protein with filtering criteria having X_{corr} scores ≥ 1.5 for singly charged, X_{corr} scores ≥ 2.0 for doubly charged and X_{corr} scores ≥ 2.5 for triply charged.

5.3 RESULTS AND DISCUSSION

5.3.1. Arsenic Effect on Cell Growth

The first goal was to delineate the effect of arsenate on cellular growth as a response to arsenic stress. Arsenate was added to growth medium at the beginning of the assay and

cell growth was monitored on a daily basis for 6 days. The results showed that at concentrations between 10 – 100 μM no significant growth inhibition was observed (not shown). However, the number of live cells, identified by moving flagella and a larger size than the dead cells, who formed clusters, began to decline at concentrations greater than 100 μM indicating that *Chlamydomonas* was unable to sustain growth in the presence of arsenate at doses above this level (Fig. 21).

The results are consistent with a previous study which showed that cell growth was stimulated in medium containing arsenic at a low concentration of 1.0 μM but was inhibited at higher concentrations (Kaise et al., 2005), despite the fact that inorganic phosphate was present in the culture medium, which would act as a competitor of arsenate in transport and binding to target molecules (Meharg and Hartley Whitaker, 2002). *C. reinhardtii* is thus incapable of detoxification of arsenate. It is possible that the inorganic form of arsenic is methylated to form monomethyl- or dimethyl arsenic such that the potential toxic effects of arsenic are increased, resulting in reactive oxygen species and formation of anti-oxidants (Meharg and Hartley Whitaker, 2002; Cao et al., 2004).

5.3.2. Extraction of soluble proteins

The accuracy of 2-D gel based proteomic analysis depends on the yield and cleanliness of the protein extract which ultimately results in protein gels with a high resolution and maximum number of proteins visible. Initially, several commonly used methods for extraction of proteins from *Chlamydomonas* were applied such as described by Förster et al. (2006) using acetone and 10% trichloroacetic acid (TCA) or as described by Gillet et al. (2006) using

streptomycin sulfate precipitation followed by a standard acetone –TCA precipitation, or polyethylene glycol (PEG) fractionation as modified used for sunflower proteins (Walliwagedara et al., 2010a).

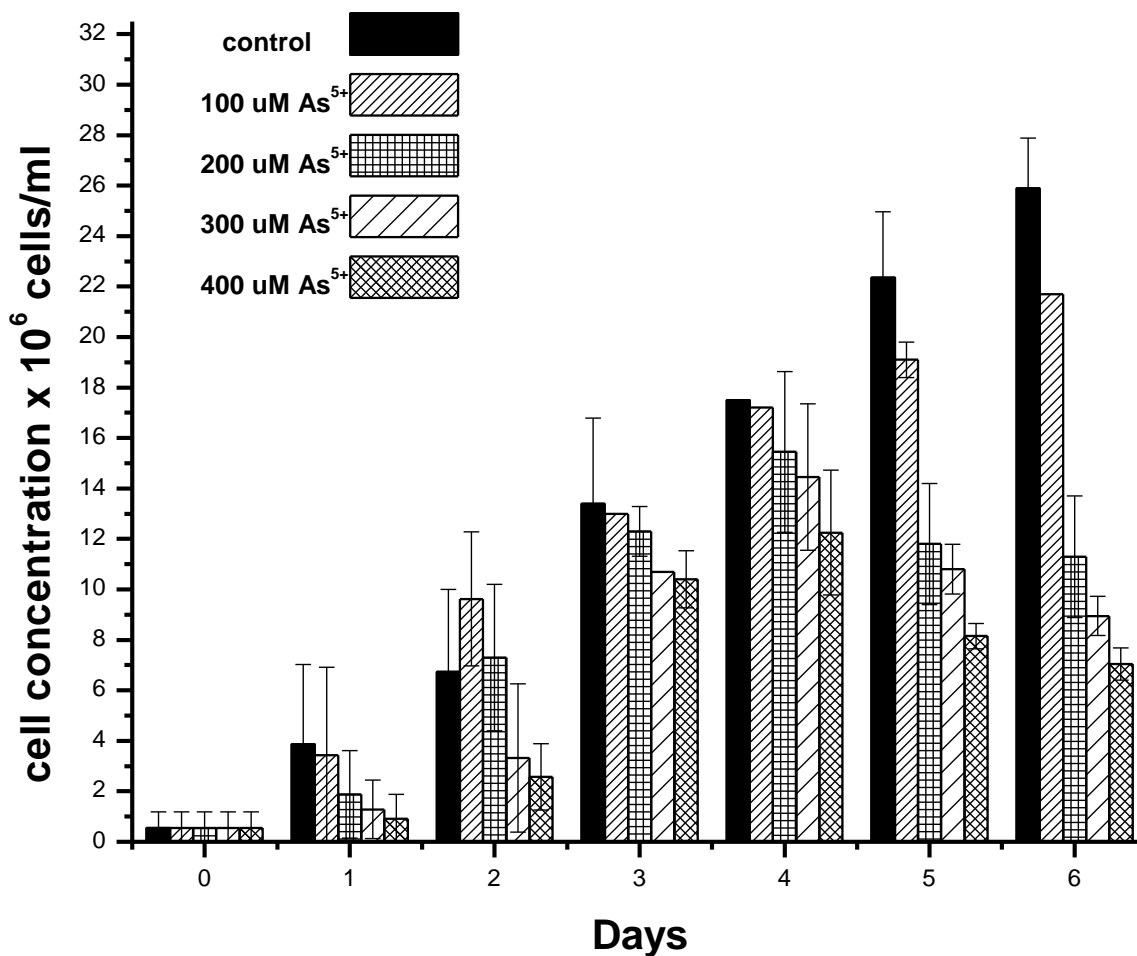
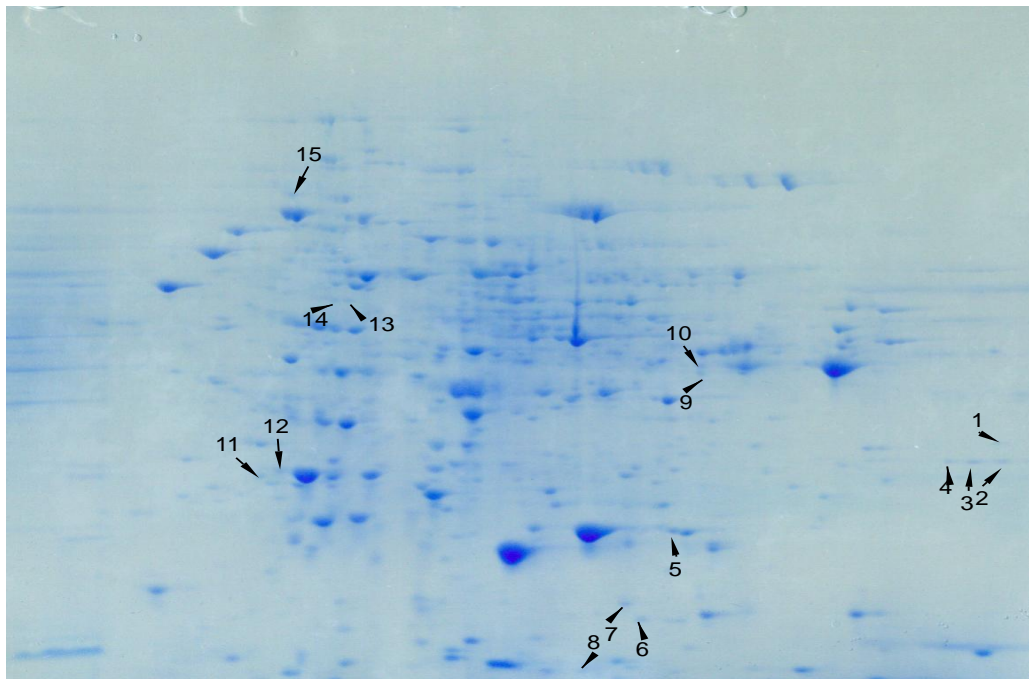
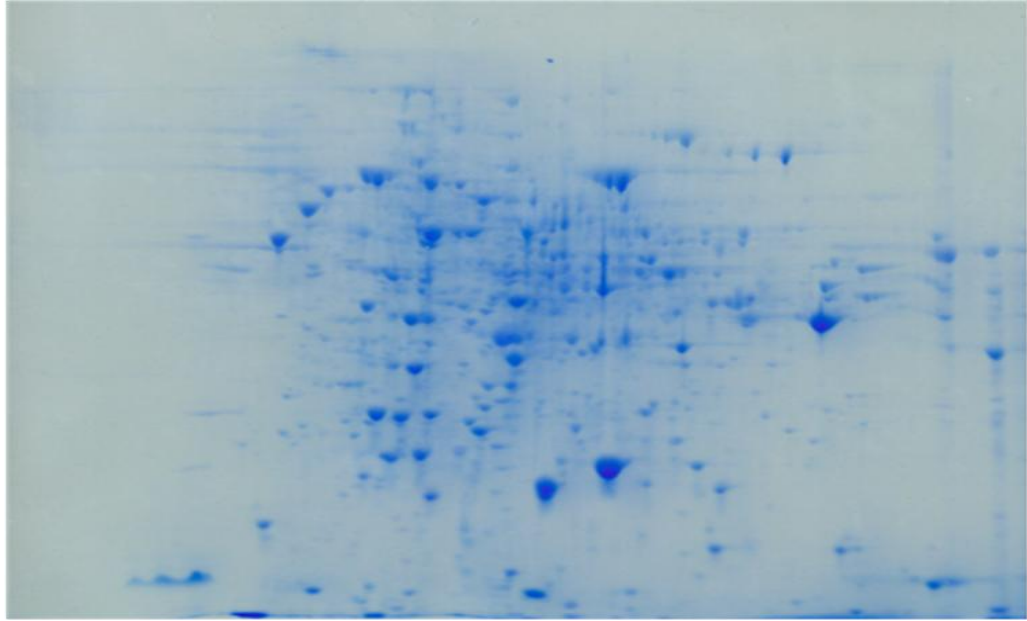


Figure 21: Effect of different arsenate concentrations on *C. reinhardtii* cell growth.

The variation in the cell growth of *C. reinhardtii* upon exposure to increasing concentrations (0, 100, 200, 300 and 400 μ M) of arsenate over 6 days of incubation at 25 °C temperature and continuous illumination was measured and expressed in live cell number per ml culture. Each assay was done in triplicate. The bar graph shows the standard deviation for each assay.

While all of these methods are commonly used to prepare proteins for 2-D gel electrophoresis, it was found that these procedures yielded gel patterns containing a considerable degree of heavy streaks with a fairly low amount of clearly visible protein spots (<100) (not shown). The low protein recovery and poor 2-D gels may be attributed to the presence of substances that coalesced soluble proteins, which was evident by the viscous nature of the final sample preparation and the color of which was always greenish. The heavy streaks could be due to the overabundance of proteins such as ribulose biphosphate carboxylase oxygenase (RuBisCO). These streaks overshadow other lower abundant proteins with similar molecular mass and isoelectric points. Removal or reduction of the streaks would thus help to identify those proteins that are differentially expressed. Therefore, another protein precipitation technique employing ammonium sulfate precipitation was tested. Ammonium sulfate precipitation has long been used in protein purification but the approach is not considered a desirable method for extracting proteins from plants, most likely due to the inability to obviate the problems associated with high protease activities in plants and the presence of such compounds as polyphenols. The protocol consisted of treatment with two consecutive salt concentrations: 40% salt treatment, followed by an increase to 60%. The first salt treatment was effective in removing substances which contributed to both the greenish color and viscosity. In addition it was found, using gel electrophoresis, that RuBisCO was selectively removed, but not many other proteins (results not shown), in a similar manner as precipitation of Sunflower RuBisCO with polyethylene glycol (Walliwalagedara et al., 2010a).



pH 3

pH 10

Figure 22: 2-D gel analysis of *Chlamydomonas* proteins.

Top panel: 2-D gel of $(\text{NH}_4)_2\text{SO}_4$ -precipitated proteins from control *C. reinhardtii*. Proteins were separated in the first dimension on an IPG strip pH 3-10 (from left to right in the figure) and in the second dimension on a 12% polyacrylamide SDS gel. A total of 500 μg protein from a 6 day culture was used. The gel was stained with GelCode Blue. **Bottom Panel:** 2-D gel of $(\text{NH}_4)_2\text{SO}_4$ -precipitated proteins from 200 μM arsenate treated *C. reinhardtii*. The sample was treated in the same way as the control. Fifteen protein spots were differentially expressed and identified using LC-MS/MS, shown by arrows.

Table X: Overview of identified proteins resulting from arsenate stress

Spot ID	<i>C. reinhardtii</i> v4.0 Acc No.	NCBI Acc. No.	Identified protein	Peptide sequence coverage %	Mol. mass kDa/pI
01	183620	XP_001689587	20S proteasome α subunit D type 7	11	27.5/8.49
02	417127	XP_001695072	Predicted protein	2	130/5.10
03	129012	XP_001690220	HLA8/High light induced nuclease	10	29.5/9.03
04	129012	XP_001690220	HLA8/High light induced nuclease	14	29.5/9.03
05	193511	XP_001699077	Superoxide dismutase[Mn]	2	24.8/7.9
06	152648	XP_001699503	Predicted protein	20	18.4/6.62
07	34270	XP_001695474	Peptidyl-prolyl cis-trans isomerase	39	22.7/9.49
08	82986	XP_001702409	RuBisCO small subunit 1	31	20.8/9.36
09	318697	XP_001697872	Hypothetical protein CHLREDRAFT_120683	30	38.6/7.0
10	318696	XP_001697872	Hypothetical protein CHLREDRAFT_120683	15	38.7/7.59
11	187228	XP_001696613	14-3-3 Multifunctional chaperone protein	37	28.1/4.94
12	130316	XP_001694699	Oxygen evolving enhancer protein 1	35	30.7/8.26
13	173725	XP_001694304	Alpha-amylase	11	44.9/6.2
14	58178	XP_001694304	Alpha-amylase	11	44.9/6.2
15	97057	XP_001695264	Heat shock protein 90B	10	80.9/4.99
A	182933	XP_001690591	Superoxide dismutase[Fe]	5	25.8/9.34
B	140618	XP_001703200	Glyceraldehyde 3-phosphate dehydrogenase	18	34.2/6.25

An increase in salt concentration to 60% resulted in precipitation of proteins whose amount was 90 % of the original protein concentration in crude extracts. The 2-D gel patterns obtained from the use of ammonium sulfate precipitated samples showed much improved

protein resolution with significantly higher number of protein spots and a substantial reduction of streaks (Fig. 22). Further, the 2-D gel patterns were highly reproducible.

5.3.3. Proteomics

Protein extracts prepared from control and arsenic-treated cells were compared to determine differential responses to As-stress. Proteins were extracted from the algae at the end of the 6th day of the incubation during which measurable decline in cell density began to occur. In the gel electrophoresis step, separation of proteins was achieved by isoelectric focusing wherein proteins were distributed in the pI range of 3 – 10. In the second dimension, SDS-polyacrylamide gel electrophoresis was performed and the gels were stained with GelCode Blue (Fig. 22). The spots that disappeared or whose intensity decreased by the arsenic treatment were not considered for identification, because they could be down-regulated proteins or represent loss of proteins resulting from degradation by cell death. Fifteen spots (1 – 15) represented over-expressed proteins when cells were grown in the presence of 200 μ M arsenate (Fig. 22, bottom panel). When cells were grown in 300 or 400 μ M arsenate more protein spots disappeared probably due to the high degree of cell death (not shown), therefore, 400 μ M arsenate was used as the maximal concentration. Usually 100, 200 and 300 μ M arsenate is used in experiments with the arsenic hyperaccumulator *Pteris vittata* (Srivastava et al., 2005), thus 400 μ M arsenate was tested as the maximal possible concentration. However, two proteins were strongly up-regulated after treatment with 400 μ M arsenate and extracted for analysis. These proteins are marked A and B in Table X.

The up-regulated proteins can be categorized on the basis of their putative functions. Of the proteins with known functions, most are associated with the removal of damaged proteins, oxidative stress, increased energy demand, protein synthesis and protein folding. The remaining proteins were annotated in the *C. reinhardtii* database as unknown or hypothetical without specific functions.

One of the up-regulated proteins is the α subunit D of the 26S proteasome (spot 1), which is part of the 20S core complex (Salomons et al., 2010). The 26S proteasome is the proteolytic complex in eukaryotes responsible for the removal of short-lived or abnormal intracellular proteins (Fu et al., 1998). The molecular organization of the 26S proteasome from the plant *Arabidopsis thaliana* has been described in some detail (Fu et al., 1998). It is structurally and functionally conserved among eukaryotes, suggesting that the subunit arrangement of the 26S proteasome in *C. reinhardtii* is probably similar to those determined for higher plants. The function of the proteasome in plants appears to be affected by external stress resulting in an increase in the core 20S proteasome at the cost of the 26S proteasome, which has a number of additional proteins. The increase of the 20S core unit appears to result in an increase in protein degradation of oxidized proteins and so enhances tolerance towards oxidative stress (Kurepa et al., 2009). The expression of this 20S proteasome subunit might be increased in the As-treated cells in order to remove proteins that are damaged by exposure to arsenate. However, no other proteasome proteins were up-regulated, so the biosynthesis of proteasome proteins might be the result of a loss of a controlling mechanism. Proteasome assembly can be impaired when one of the lid proteins is absent as was found in fission yeast (Salomons et al., 2010). It is also possible that the other core proteins were not detected in the 2-D gels.

Another protein identified in these experiments is Mn-dependent superoxide dismutase (SOD) (spot 5), which catalyzes the reduction of superoxide anions to hydrogen peroxide. The increase of this enzyme might be to reduce oxidative stress, which results in production of reactive oxygen species, an observation that also was made using the Dwarf Sunflower (Walliwalagedara et al., 2010b), a similar observation was made when *Chlamydomonas* was exposed to Cd (Gillet et al., 2006). When cells were exposed to 400 μ M arsenate a different SOD was identified namely a Fe-dependent SOD (spot extra 1). Studies on maize, for instance, have indicated that an increase in antioxidant capacity improved tolerance to arsenic Srivastava et al., 2005). Another protein expressed under arsenic stress is peptidyl-prolyl cis-trans isomerase (spot 7), which catalyzes the interconversion of peptidyl-prolyl imide bonds in peptides and proteins. It is also expressed in higher plants in response to oxidative stress (Shapiguzov et al., 2006). In addition, prolyl isomerases act together with chaperonins, and protein disulfide isomerases that are involved in protein folding (Göthel and Maraheil et al., 1999). The chaperonin proteins are expressed in hyperaccumulator plants when exposed to arsenic and other toxic metals (Ahsan et al., 2009). In the present study, one spot was identified as a chaperonin having multifunctional activities (spot 11). Chaperonins appear to have diverse functions including protein synthesis, folding, and posttranslational modification. During metal stress they are able to prevent irreversible protein denaturation or help channel proteins for proteolytic degradation (Sarry et al., 2006). Recently, this protein has been identified and characterized in higher plants but its precise role in plant cells remains unclear. It may be that the expression of this protein plays an essential role in the removal of damaged or abnormally folded proteins. Heat shock protein 90B that was expressed as spot 15 is also a chaperonin protein and it has been

reported to be expressed under metal stress (Aly et al., 1994, Feder and Hofmann, 1999). Previously it was shown that a heat shock protein was up-regulated in Dwarf Sunflowers upon exposure to lead (Walliwalagedara et al., 2010b).

Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) (spot 8) is a major enzyme in green plants that catalyzes carbon assimilation, hence controls photosynthetic rate and thus has a major effect on plant growth. The increased expression of the RuBisCO small subunit could be to compensate for damage to the photosynthesis system. Degradation of RuBisCO in metal non-tolerant plants has been reported in response to redox-reaction associated heavy metals such as copper and cadmium and other metals including mercury, cobalt, manganese and zinc (Führs et al., 2008, Hajduch et al., 2001, Kieffer et al., 2009) and has been described in previous chapters. A down-regulation of RuBisCO subunits, on the other hand, was observed in hyperaccumulator plants following exposure to metal stress (Tuomainen et al., 2006) and also was shown in a previous investigation using As-exposed Dwarf Sunflowers see chapter 3, suggesting a decrease in net photosynthesis under metal stress. However, it is more likely that there is no down-regulation but degradation that takes place as shown in the previous chapters. However, the large subunit of RuBisCO was not detected and could, therefore, not be quantitated because it was removed by the ammonium sulfate precipitation. Treatment of *Chlamydomonas* with Cd resulted in a down-regulation of the large subunit; the small subunit was not identified as up- or down- regulated in this particular study (Gillet et al., 2006).

The increase of expression of α -amylase (spot 13 and 14) is indicative of an increase in energy requirement, which maybe the result of damage to the alga's photosynthetic capacity. It must be noted that the cells were growing in TAP medium, which contains

acetate that can be used as a carbon source. Under similar conditions using Cd, it was found that enzymes in the glyoxylate pathway (isocitrate lyase) and in glycolysis/gluconeogenesis (phosphoglycerate mutase) were up-regulated (Gillet et al., 2006). Treatment with As had a different effect, but at 400 μ M As, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (spot B) was strongly up-regulated, supporting the possible increase in gluconeogenesis or simply recycling of carbon compounds via the C2 recovery pathway. However, it has been shown that GAPDH is a pleiotropic protein involved in various stresses, programmed cell death, DNA damage repair and DNA replication (Kim and Dang 2005). Differential expression of GAPDH in response to metal stress has been observed in several proteomic studies (Kieffer et al., 2009, Ahsan et al., 2008, Fukuda et al., 2007), and might not be related to energy metabolism at all but to any of the above mentioned effects.

Oxygen evolving enhancer protein 1 (OEE) (spot 12) is another protein expressed under stress (oxygen radicals and heat). It plays a major catalytic role in photosynthetic oxygen evolution in green plants, and is associated with the photosystem II complex, the site of oxygen evolution in all higher plants. The OEE of photosystem II also was shown to exhibit thioredoxin activity in *C. reinhardtii* (Heide et al., 2004, Rolland et al., 2009) and it has been reported that these genes of *C. reinhardtii* are expressed upon exposure of cells to high light and low CO₂ concentrations (Im and Grossman, 2001). The dual role of OEE 1 as a thioredoxin in metabolism and a function in light-driven electron flow (Heide et al., 2004) is supported by the increase of its synthesis during arsenate-induced stress and the overall importance of maintenance of the proper redox state.

5.4 CONCLUSION

In conclusion, the response of *C. reinhardtii* is different as found for Sunflowers, there is no general defense mechanism. The majority of the cell's reaction appears mainly to be a response to protein and oxidative damage. However, the similarity to Sunflowers is seen in the increase of some metabolic enzymes, which suggests that photosynthesis and sugar metabolism is impaired. Other enzymes might be up-regulated as well but are below the detection limit. No specific As-binding proteins were found as was one of the premises of this project. As a final conclusion it can be stated that it appears that *C. reinhardtii* might not be a good model to study the response to As uptake.

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CHAPTER 6

CHINESE BRAKE FERN *PTERIS VITTATA*: ARSENIC UPTAKE KINETICS AND DIFFERENTIAL PROTEOMIC ANALYSIS IN RESPONSE TO ARSENIC EXPOSURE

6.1 INTRODUCTION

Arsenic compounds are widespread mainly due to various man-made activities and contamination in water and soil is a major health problem, for they are toxic to most living systems including humans, animals, and plants (Fowler, 1983). Environmental sources of arsenic stem from the use of its compounds as pesticides, during the mining and smelting of heavy metals, and burning of high arsenic coals (Smith et al, 1998). Consumption of contaminated drinking water has led to a global epidemic of arsenic poisoning with increased occurrences of cancer and Blackfoot disease in Taiwan, Argentina, Chile, and Bangladesh (Bei et al., 2001; Tripathi et al., 2007; Tsai and Liao 2006; Ultra et al., 2007). The toxic forms of As include arsenite (AsO_2^-), which is reactive towards thiol groups and acts on proteins and cofactors (lipoic acid) and arsenate (AsO_4^{3-}), which is an analogue of phosphate

and interferes with phosphorylation and thus energy metabolism (Poynton et al., 2004). Arsenate is apparently transported by phosphate transporters and reduced to arsenite in plants by an arsenate reductase (Singh and Ma, 2006).

Remediation of arsenic contaminated soils and water have therefore become a major environmental issue. The most well-known As hyperaccumulators are typically members of the order of Pteridales: *Pteris vittata* (Chinese brake fern) and *Pityrogramma calomelanos* (silver fern) (Poynton et al., 2004; Wei and Chen, 2006; Zhang et al., 2002). Other ferns with the ability to hyperaccumulate As include: *P. ensiformis*, *P. umbrosa*, *P. multifida*, *P. cretica* and *P. argyraea* (Baldwin and Butcher, 2007; Gonzaga et al., 2006; Koller et al., 2007; Wang et al., 2007). *Pteris vittata* (Ma et al., 2001) can accumulate arsenic as high as 23,000 mgkg⁻¹ in their fronds when growing in a soil spiked with 1500 mgkg⁻¹ As. Different aspects of As hyperaccumulation in *P. vittata* have been discussed by several groups including uptake kinetics, root to shoot transport, interactions with phosphate, As speciation (Wang et al., 2002, Ze-chun et al., 2007), and antioxidative responses upon As accumulation (Cao et al., 2004, Srivastava et al., 2005). The most striking feature is the highly efficient transport of As from roots to fronds. For example it was reported that 76% of the As taken up by *P.vittata* was transported to its fronds within 8 h of arsenate exposure (Mc Grath et al., 2002). Other mechanisms that allow dealing with elevated levels of arsenic appear to be related to the induction of the enzymes that counteract oxidative stress, although it appears that *P. vittata* sequesters As so efficiently that oxidative stress is less than found in other plants. The discovery of an arsenic transporter that moves arsenic into vacuoles explains the fern's high tolerance to arsenic. A proteomic analysis of *P. vittata* has shown that protein expression in fronds under chronic, non-lethal, amounts of As is influenced by arbuscular

mycorrhizal symbiosis and showed a central role of glycolytic enzymes in arsenic metabolism.

Previously, it was found in hydroponically-grown dwarf sunflowers using a proteomics approach that oxidative stress is one of the major effects of As, see Chapters 3 to 5. The aim of this study was to see if *Pteris vittata* showed a similar response when grown hydroponically and if the amount of arsenate added makes a difference in the ferns' response.

6.2 METHODS AND MATERIALS

6.2.1. Plant growth and arsenic treatment

Four weeks old *Pteris vittata* ferns were purchased from Milestone Agriculture, Inc (Apopka, FL) and were transferred into medium-sized pots containing general purpose gardening soil and were allowed to grow to four to five healthy fronds under greenhouse conditions. Healthy plants were then transferred into Deep Water Culture (DWC) basic hydroponic setups (Stealth Hydroponics, Saline, MI) containing water mixed with required amounts of essential macro- and micro-nutrients (Stealth Hydroponics, Saline, MI). Plants were allowed to grow 2-3 weeks until they were established in a hydroponic environment. Hydroponic setups were allocated and labeled for control and arsenic-treated experiments. Experiments were conducted to establish the toxicity ranges using potassium dihydrogen arsenate (KH_2AsO_4) and concentrations were varied from 150 to 600 μM . Two plants having 4-5 fronds in each were used for each experimental setup. The morphology of the fronds was

observed everyday up to seven days and the plants were harvested at the end of the seven days growth period. Fronds and roots were separated and wet weights were recorded as a total biomass for each experimental setup. The fronds and roots were stored at -80°C or immediately processed for either extraction of total proteins or arsenic uptake studies. Each experiment was repeated twice with independently grown plants.

6.2.2 Determination of Arsenic Uptake

Two plants having 4-5 fronds in each were used for each experimental setup and only the concentrations of 150 and 300 μM of arsenate (KH_2AsO_4), arsenite (NaAsO_2) and Dimethylarsenic acid (DMA)/ (CH_3)₂AsO₂H) were used for metal uptake experiments. Plants were grown hydroponically in DWC basic hydroponic setups and arsenic solutions were spiked accordingly. All the experiments were repeated three times. Plants were harvested, sectioned into fronds and roots and dried in an oven at 60°C until a constant dry weight was observed. The dried plant materials were ground using liquid N_2 and the powder was directly used for the sample preparation process for Inductively Coupled Plasma Spectroscopy (ICP). The digestion procedure Method 3050B was obtained from The US Environmental Protection Agency (USEPA). The final extracts were subjected to ICP with standard series of arsenate,, arsenite and dimethyl arsenic acid respectively and the amount of atomic arsenic taken up by fronds and roots were measured.

6.2.3. Sample Preparation for Gel Electrophoresis

After seven days of growth under experimental conditions plants were harvested and fronds and roots were separated, weighed (wet weight) and were ground to a fine powder in liquid N₂. Protein extraction was performed using Polyethelene glycol (PEG) fractionation described by Xi et al. (2006). Some modifications were made in order to minimize the loss of proteins as described below. Dry tissue (0.2 g per tube) was homogenized in 1 ml of ice cold protein extraction buffer (0.5 M Tris.HCl, pH 7.8, 2% (v/v) NP-40, 20 mM MgCl₂, 2% 2-ME (v/v), and Complete Protease Inhibitor cocktail (G-Biosciences), 1 mM EDTA and 1 % (w/v) polyvinylpolypyrrolidone (PVPP)). The cell free slurry was prepared by sonication and mechanical grinding. It was centrifuged at 12,000 x g at 4° C for 15 min and the pellet was labeled as *F1*. The supernatant was then subjected to PEG fractionation. To the supernatant, 50 % (w/v) PEG (PEG 4000) stock solution was added to give a final PEG concentration of 16 %. The mixture was incubated in an ice bath for 30 min and centrifuged (12,000 x g at 4° C for 15 min and the pellet was labeled as *F2*. The supernatant was mixed with four volumes of cold 10 % TCA/acetone with 0.07 % 2-ME and incubated overnight at -20° C. After the centrifugation (15,000x g, 4° C, 10 min) the resulting pellet gave rise to fraction 3 (*F3*). All pellets were washed with ice-cold acetone with 0.07 % 2-ME, vacuum dried and stored at -80 ° C.

6.2.4. Two-Dimensional Protein Gel Electrophoresis

Protein pellets were resuspended in freshly prepared rehydration buffer (RB) (7 M Urea, 2 M thiourea, 1% CHAPS, 1% Triton X-100, 1% DTT and 0.2% Ampholytes and 1%

BromoPhenolBlue). A total of 500 µg of protein was used for 2-DE analysis. Each pellet (Fraction 3/F3) was resuspended in 200 µl of rehydration buffer at room temperature. Rehydration was performed by loading the total 200 µl RB on 11 cm pH 3-10 linear immobilized pH gradient (IPG) strips (BioRad, Hercules, CA) into the tray. Active rehydration was performed at 5 V for 14-16 h at room temperature. The IPG strips were focused using a Protean IEF cell (Bio-Rad) according to the manufacturer's instructions and the total volt-hours reached at the end of IEF was 40 kVh. After isoelectric focusing was completed, proteins were separated using a 12% SDS-PAGE gel (Bio-Rad) at a voltage of 200 V. The gels were fixed in a 100 ml fixing solution (50 % ethanol, 10% acetic acid in water) for 30 min at room temperature with gentle shaking. After washing with water, the gels were stained overnight with Gel Code Blue staining solution (Pierce, Rockford, IL).

6.2.5. Image Analysis of Two-Dimensional Gels

Gels were scanned at 300 ppi on a Scan maker, 5900 scanner, (MicroTek Lab Inc, CA) and the scanned images of protein spots were loaded into the PDQUEST gel analysis software (Version 6.2, BioRad). The data set was obtained from gels using proteins from control and arsenic-treated cells. The proper spot detection parameters were selected and adjusted using the Spot Detection Wizard in order to identify and count all the protein spots of interest in the gels. Match sets were created using all gels and up-regulated/over-expressed spots at different concentrations of arsenic exposure were determined using a Student's t test within PDQUEST with a significance level set to 95% ($p < 0.05$). Replicates from three independent extracts were used for analysis.

6.2.6. In-gel Tryptic digestion

Stained protein spots that appeared as up-regulated after As treatment were selected and excised manually. They were cut into smaller pieces and transferred to 1.5 ml microcentrifuge tubes. The excised gel fragments were washed twice for 1 h each with 175 μ l of wash reagent (50 % ethanol (95% USP grade), 5% acetic acid (99.9% Aldrich) and water) to remove the dye that was bound to the protein. It was then dehydrated for 5 min using 175 μ l of HPLC grade acetonitrile (Burdick and Jackson, NJ) and rehydrated for 5 min with 100 mM ammonium bicarbonate. It was again dehydrated for 5 min with acetonitrile and vacuum-dried in a SpeedVac for approximately 3 min. After this dehydration/rehydration procedure, the gel pieces were digested with trypsin by adding 10-15 μ l of 10 ng/ μ l trypsin reagent (Promega sequencing grade modified trypsin dissolved in 2 ml 50 mM ammonium bicarbonate). The gel pieces were vortexed, centrifuged briefly and digested overnight at room temperature. The resulting peptides were extracted from the polyacrylamide in two aliquots of 30 μ l of extraction reagent (50% acetonitrile, 5% ACS grade formic acid). These extracts were combined and evaporated to less than 10 μ l in a SpeedVac and then resuspended in 1% acetic acid to make a final volume of approximately 30 μ l for LC-MS/MS analysis.

6.2.7. Mass Spectrometry Analysis

The mass spectrometric analysis for the identified protein spots from the treated gels were performed using either LCQ-Deca or LTQ Linear ion trap mass spectrometer (ThermoScientific, San Jose, CA) located in the Proteomics Core, Lerner Research Institute, the Cleveland Clinic, Cleveland, OH. The HPLC column was a self-packed 10 cm x 75 μ m

Phenomenex Jupiter C18 reversed-phase capillary chromatography column. One to ten μl of the tryptic digests were injected and the peptides were eluted from the column by an acetonitrile / 0.05 M acetic acid gradient at a flow rate of 0.2 $\mu\text{l}/\text{min}$. The nanoelectrospray source was operated at 2.5 kV and the mass spectrometer was operated in a data-dependent mode.

6.2.8. Protein Identification

Since there is no *P. vittata* data base available for the protein identification, a reference data base was created using NCBI plant reference sequence. The LC-MS/MS data (from Xcalibur raw files) for each sample was searched against the above mentioned Plant protein reference sequence database and additional searches were performed using the full NCBI non-redundant database (The Genetic sequence database at the National Center for Biotechnology Information). Protein versus protein alignment searches were performed for each protein sequence as well as for the peptide sequences obtained. The identity of these proteins was further proved by SEQUEST, tandem mass spectrometry database searching algorithm on Bioworks software (Thermo Finnigan). All the MS/MS spectra were searched against the databases generated from identified protein sequences for each protein with filtering criteria having X_{corr} scores ≥ 1.5 for singly charged, X_{corr} scores ≥ 2.0 for doubly charged and X_{corr} scores ≥ 2.5 for triply charged.

6.3 RESULTS AND DISCUSSION

6.3.1 Arsenic Uptake Kinetics

P. vittata is known as an arsenic hyperaccumulator fern which shows an extraordinary high capacity to tolerate high concentrations of arsenic without showing any phytotoxic effects. It was first identified as an As accumulator by Dr. Lina Q. Ma of the Soil & Water Science Department at the University of Florida (Ma et al., 2001). The fern can accumulate As up to 27,000 mg/Kg dry weight in its fronds though the phytotoxic symptoms started to appear once the As concentration exceeds approximately 10,000 mg/Kg dry weight (Wang et al., 2002, Tu and Ma, 2002) which is a much higher number compared to many other non-hyperaccumulator plant species, which have threshold values between 5-100 mg/Kg dry weight. The elucidation of the biochemical mechanism of As hyperaccumulation in *P. vittata* has become very challenging and most of the research groups have worked on its arsenic uptake kinetics, interaction with phosphate and arsenic speciation and very few studies have been reported on proteomic approaches to understand the hyperaccumulation mechanisms. Therefore, this study was mainly focused on the differential expression of proteins in responses to arsenic exposure and to see whether the proteomic approach is a useful means to understand the biochemical mechanisms of As hyperaccumulation in *P. vittata*. Initial experiments were performed in order to understand the uptake kinetics of As by varying the concentration and species of As used. Two different concentrations were used: 150 and 300 μM and 3 species of arsenic: arsenate (KH_2AsO_4), arsenite (NaAsO_2) and Dimethylarsenic acid (DMA) ($\text{CH}_3)_2\text{AsO}_2\text{H}$). Several research groups have shown that the

arsenic concentration increases in plants when exposed to arsenic and that the fronds are the storage sites that can accumulate most of it (Ma et al., 2001). This was again discussed by Tu et al., (2002) showing that As concentration in the fronds rapidly increased from 12.1 mg/Kg when the plants were transplanted to 6,000 mg/Kg at the eighth week after transplanting. The As concentration eventually reaches to a higher but constant concentration level with time. On the contrary roots accumulate a 20 times lower arsenic concentration (Tu et al., 2002). The experiment presented here, see Figure 23, also showed that fronds have the highest concentration of As compared to that of roots and that it is only 6-10 times lower in the roots which might be due to a shorter exposure time compared to that used by of Tu et al. (2002) who used a 20 week exposure time. The experiment shown in Figure 1, indicates that the concentration of As in fronds and roots were directly proportional to the As concentrations supplied in the growth medium and the highest concentrations were found in both fronds and roots when the plants were treated with the highest concentration used: 300 μ M. In addition, there is no large difference in the values of plant As concentrations (fronds or roots) when arsenate versus arsenite treatment were compared, but a comparatively higher concentration in both fronds and roots was observed when the ferns were exposed to DMA. A similar result was shown by Kertulis et al., (2005) although these authors used a different concentration range.

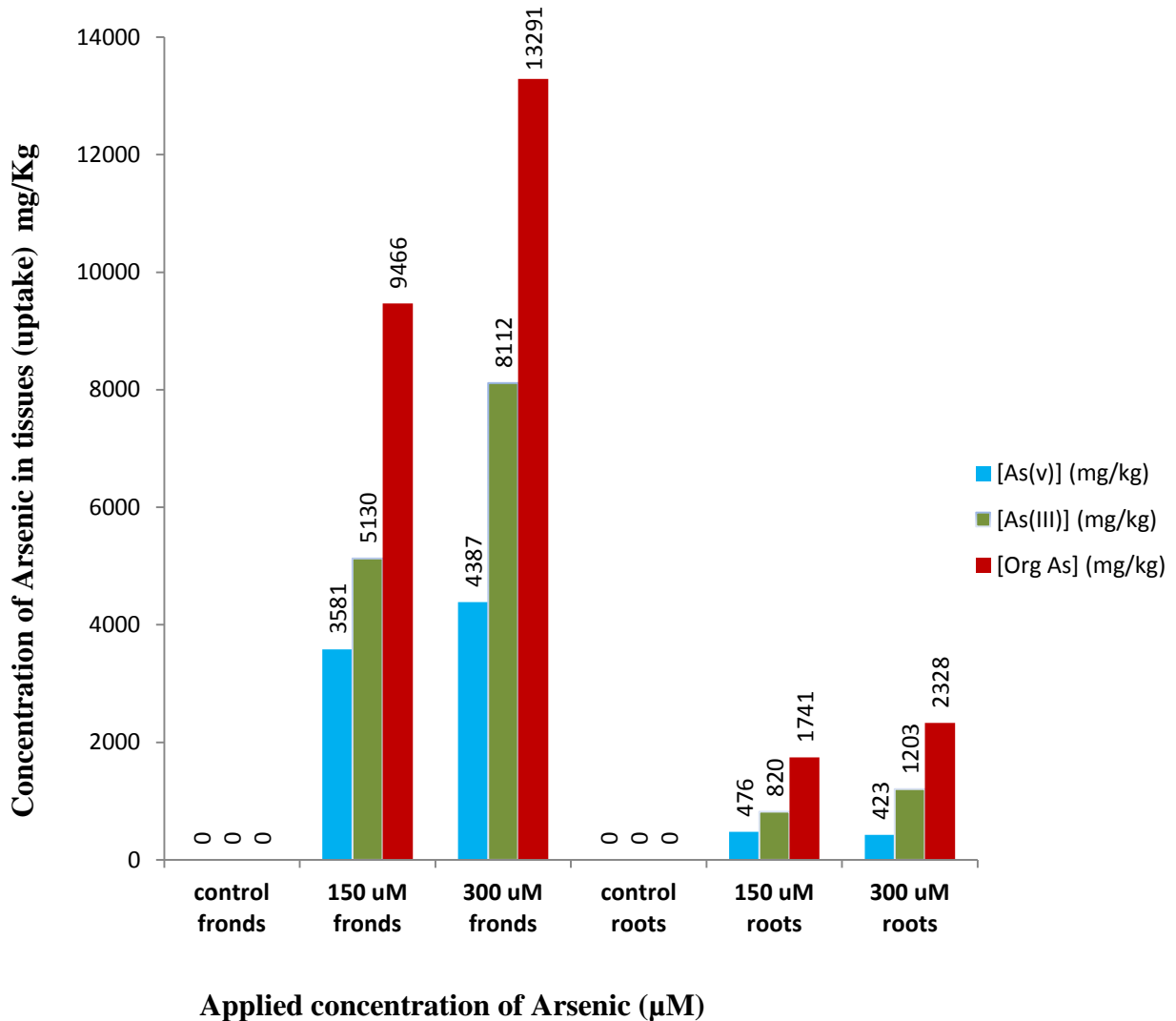


Figure 23: Total atomic arsenic concentrations in the fronds and roots of *P. vittata*

P. vittata plants were grown in a hydroponic system and were exposed to 150 and 300 μM arsenic as arsenate (KH_2AsO_4) (blue), arsenite (NaAsO_2) (green) or Dimethyl arsenic (DMA) (red). The average of three measurements is indicated in the Figure. Arsenate, arsenite or DMA were detected as atomic arsenic. The oxidation state was not recorded.

The experiment presented in Figure 23 shows that the level of arsenic in roots is higher when plants were treated with organic arsenic as compared to ferns treated with inorganic arsenic. The highest As concentration in fronds also was observed when ferns were

treated with organic As: it is 2 times higher than As (III) and 3 times higher than As (V). There is no difference in the pattern between treatment with 150 or 300 μM of the toxins.

Several research groups (Zhang et al., 2002; Tu et al., 2003; Webb et al., 2003) have reported that when plants are grown on arsenate in the soil, they convert it to arsenite. The results presented in Figure 23 show that arsenite can also be taken up as well as organic arsenic. Since ICP only measures atomic arsenic, the oxidation state or arsenic modification could not be determined. It is possible that arsenate and DMA are converted to arsenite, however, it is interesting that the plant takes up arsenite and DMA. Plants prefer to take up arsenate which is a phosphate analog thus phosphate transporters are used and arsenate is subsequently transported to fronds (Poynton et al., 2004, Wang et al., 2002). Once As enters into the fronds, As is localized in the vacuoles of the upper and lower epidermal cells and trichomes (Lombi et al., 2002). Su et al., (2008) found that the form of As translocated in xylem is actually arsenite and the roots are the main location of arsenate reduction in *P. vittata* by an arsenate reductase (Su et al., 2008; Indriolo et al., 2010).

6.3.2 Proteomics

Proteomic studies were performed using arsenate and the concentration range to which the ferns were exposed to was 150, 300, 450 and 600 μM for seven days. Bona et al. (2010) used for a proteomics study 330 μM arsenate for 60 days and refer to it as chronic arsenic contamination in the soil. Another experiment was performed by Singh et al. (2006) using hydroponically grown ferns with 133 and 267 μM arsenate for ten days maximally.

Thus the amounts used are within the range used by others, with 600 μM as an example for high toxicity.

As described in chapter 2, PEG fractionation was used for protein sample preparation from Fern fronds for 2-DE because it worked the best for *Helianthus annuus*. The obtained 2-DE profiles of *P. vittata* proteins isolated also were of good quality and the spots were well resolved. The result of protein separation by 2-DE is shown in Figure 24.



Figure 24: 2-DE gel of proteins isolated from control *P. vittata* fronds.

Proteins isolated from fronds were subjected to PEG fractionation and a total of 500 μg of protein (Fraction 3/F3) was separated in the first dimension on an IPG strip pH 3- 10 (from left to right in the Figure) and in the second dimension on a 12 % polyacrylamide gel in the presence of SDS. The gel was stained with GelCode Blue.

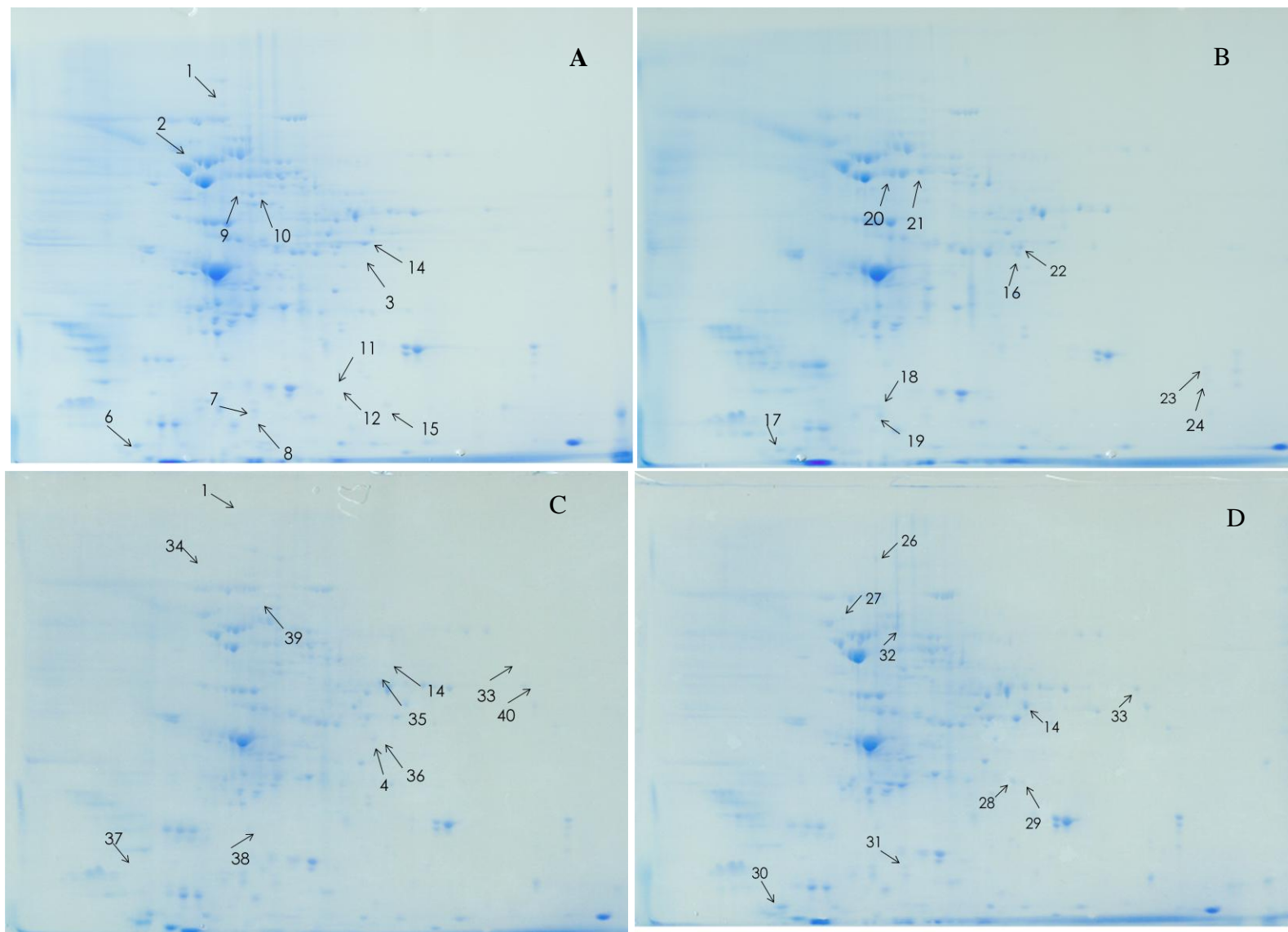


Figure 25: 2-DE gels of PEG fractionated proteins from *P. vittata*.

Proteins were separated in the first dimension on an IPG strip pH 3-10 (from left to right in the figure) and in the second dimension on a 12% polyacrylamide SDS gel. A total of 500 μg protein (Fraction 3/F3) from fronds exposed to different concentrations of arsenate were used. The gel was stained with GelCode Blue. A: 150 μM , B: 300 μM , C: 450 μM and D: 600 μM arsenate. A total of 40 proteins were differentially expressed and identified by LC-MS/MS shown by arrows.

Table XI: Identification of the selected proteins in *Pteris vittata* in response to arsenic stress

Spot No.	NCBI Acc. No.	Identified protein	150 μ M	300 μ M	450 μ M	600 μ M	Seq. cov. (%)	Species	Identified Protein	<i>Physcomitrella</i>	#of genes
1	gi 302791772	Hypothetical protein SELMODRAFT_152047	Yes	No	No	Yes	4	<i>S. moellendorffii</i>	Metalloendopeptidase	Pp1s72_282	1
2	gi 224104681	predicted protein	Yes	No	No	No	12	<i>P. trichocarpa</i>	Mitochondrial chaperonin (TCP-1/cpn60)	Pp1s16_322	2
3	gi 242041603	Hypothetical protein SORBIDRAFT_01g041500	Yes	No	No	No	1	<i>S. bicolor</i>	Tropomyosin-like DUF_1423	Pp1s15_294	5
4	gi 15240250	RPE; Catalytic/ribulose phosphate 3-epimerase	Yes	No	No	Yes	19	<i>A. thaliana</i>	RPE; Catalytic/ribulose phosphate 3-epimerase	Pp1s2_15	7
5	gi 302840943	Hypothetical protein VOLCADRAFT_75246	Yes	No	No	No	5	<i>V. cateri f. nagariensis</i>	V_ATPase_I	None	
6	gi 15233264	MATH domain containing protein	Yes	No	No	No	2	<i>A. thaliana</i>	Extracellular metalloprotease	Pp1s119_72	10
7	gi 186510270	RNA binding/NA binding/Nucleotide binding	Yes	No	No	No	10	<i>A. thaliana</i>	RNA binding/RNA recognition motif	Pp1s462_2	9
8	gi 297810087	Hypothetical protein ARALYDRAFT_490484	Yes	No	No	No	34	<i>A. lyrata subsp. lyrata</i>	Ribosomal_P1_P2_L12P	Pp1s152_152	7
9	gi 297790512	predicted protein	Yes	No	No	No	28	<i>A. lyrata subsp. lyrata</i>	predicted protein	None	
10	gi 242081967	Hypothetical protein SORBIDRAFT_07g025090	Yes	No	No	No	18	<i>S. bicolor</i>	Enolase-like (RRM superfamily)	Pp1s83_55	6
11	gi 15241166	Peroxisomal membrane 22 kDa family protein	Yes	No	No	No	12	<i>A. thaliana</i>	Peroxisomal membrane protein (Mpv17_PMP22)	Pp1s35_68	13
12	gi 225437602	Predicted:hypothetical protein	Yes	No	No	No	20	<i>v. vinifera</i>	Hypothetical protein	None	
13	gi 255088051	UBA/THIF-type NAD/FAD binding fold domain	Yes	No	No	No	4	<i>M. sp. RCC299</i>	Metallo_dep._hydrolases DNase	Pp1s391_37	11
14	gi 302774424	Hypothetical protein SELMODRAFT_227983	Yes	No	Yes	Yes	6	<i>S. moellendorffii</i>	Fructose bisphosphate aldolase class 1	Pp1s475_27	8
15	gi 302843109	Hypothetical protein VOLCARAFT_105797	Yes	No	No	No	2	<i>V. cateri f. nagariensis</i>	Vps51/Vps67 sec5 subunit excyst complex	Pp1s38_295	3

Spot No.	NCBI Acc. No.	Identified protein	150 μ M	300 μ M	450 μ M	600 μ M	Seq. cov. (%)	Species	Identified Protein	<i>Physcomitrella</i>	#of genes
16	gi 225431122	Predicted:hypothetical protein isoform 1	Yes	No	No	No	3	<i>V.vinifera</i>	NADPH flavin oxidoreductase	Pp1s131_175	15
17	gi 302845333	Hypothetical protein VOLCADRAFT_95023	No	Yes	No	No	2	<i>V.cateri f. nagariensis</i>	Hypothetical protein VOLCADRAFT_95023	None	
18	gi 303277115	predicted protein	No	Yes	No	No	4	<i>M. pusilla CCMP1545</i>	Spliceosome subunit LUC7	Pp1s250_58	5
19	gi 15238170	60S acidic ribosomal protein P1 (RPP1C)	No	Yes	No	No	35	<i>A.thaliana</i>	Ribosomal_P1_P2_L12P	Pp1s152_152	7
20	gi 226530160	Hypothetical protein LOC100278108	No	Yes	No	No	17	<i>Z.mays</i>	Hypothetical protein LOC100278108	None	
21	gi 168039562	predicted protein	No	Yes	No	No	11	<i>P. patens subsp. Patens</i>	RNA binding domain/recognition (RRM)	Pp1s148_7	41
22	gi 115484401	Os11g0171300	No	Yes	No	No	6	<i>O. sativa</i>	Fructose bisphosphate aldolase class 1	Pp1s475_27	8
23	gi 302772250	Hypothetical protein SELMODRAFT_440774	No	Yes	No	No	2	<i>S. moellendorffii</i>	Guanyl transferase eIF-2B	Pp1s144_52	5
24	gi 302836880	Hypothetical protein VOLCADRAT_90391	No	Yes	No	No	4	<i>V.cateri f. nagariensis</i>	Sorting nexin Phox homology domain	Pp1s76_78	2
25	gi 68064185	predicted protein	No	Yes	No	No	5	<i>P. patens subsp. Patens</i>	Plant protein involved in nitrogen contr. devel.	Pp1s387_8	15
26	gi 115471703	Os07g0412100	No	No	Yes	No	1	<i>O. sativa</i>	Glycosyl transferase group 1 ADP-glucose	Pp1s33_144	16
27	gi 11488160	Os12g0277500	No	No	Yes	No	2	<i>O. sativa</i>	Chaperonin_like (TCP-1/cpn60)	Pp1s170_46	4
28	gi 302853782	Hypothetical protein VOLCADRAFT_99655	No	No	Yes	No	4	<i>V.cateri f. nagariensis</i>	Ubiquitin thioesterase	Pp1s53_227	4
29	gi 302840782	Hypothetical protein VOLCADRAFT_92428	No	No	Yes	No	1	<i>V.cateri f. nagariensis</i>	Cyclic nucleotide gated channel	Pp1s204_120	16
30	gi 302770529	Hypothetical protein SELMODRAFT_409700	No	No	Yes	No	2	<i>S. moellendorffii</i>	AAA ATPase/ bromodomain/P-loop	Pp1s44_156	95
31	gi 303290106	predicted protein	No	No	Yes	No	3	<i>M.pusilla CCMP1545</i>	Multifunctional chaperone 14-3-3	Pp1s37_23	11
32	gi 168027405	predicted protein	No	No	Yes	No	8	<i>P. patens subsp. Patens</i>	Enolase_like	Pp1s83_55	6
33	gi 302774424	Hypothetical protein SELMODRAFT_227983	No	No	Yes	Yes	16	<i>S. moellendorffii</i>	Fructose bisphosphate aldolase class 1	Pp1s33_389	17
34	gi 224136294	predicted protein	No	No	No	Yes	12	<i>P.trichocarpa</i>	SAM domain, domain-domain interactions	None	
35	gi 297721039	Os02g0254800	No	No	No	Yes	3	<i>O. sativa</i>	SWIM superfamily	None	

Spot No.	NCBI Acc. No.	Identified protein	150 μ M	300 μ M	450 μ M	600 μ M	Seq. cov. (%)	Species	Identified Protein	<i>Physcomitrella</i>	#of genes
36	gi 297724845	Os06g0472600	No	No	No	Yes	9	<i>O. sativa</i>	Os06g0472600 no conserved domains	None	
37	gi 302832361	Hypothetical proteins VOLCADRAFT_87927	No	No	No	Yes	5	<i>V.cateri f. nagariensis</i>	Sec23/sec25 vesicle coat complex	Pp1s220_95	12
38	gi 297824591	Hypothetical protein ARALYDRAFT_483677	No	No	No	Yes	16	<i>A.lyrata subsp. Lyrata</i>	DUF260 super family	Pp1s35_338	31
39	gi 68066435	predicted protein	No	No	No	Yes	5	<i>P. patens subsp. Patens</i>	Leu –rich protein kinase (PkC-like)	Pp1s446_18	2
40	gi 302853782	Hypothetical protein VOLCADRAFT_99655	No	No	No	Yes	1	<i>V.cateri f. nagariensis</i>	Ubiquitin thioesterase	Pp1s72_180	2

Proteins were purified from fronds of ferns exposed to different concentrations of arsenate and subjected to 2-D polyacrylamide gel electrophoresis. The results are shown in Figure 25 for the four concentrations of arsenate used (150, 300, 450 and 600 μM). The spots marked in Figure 25 were up-regulated and detected as described in the Materials and Methods section and as final check visually authenticated. The results of analysis by mass spectrometry is summarized in Table XI. The initial screen was done automatically using the standard search protocols against the GenBank database. Matches were obtained with proteins from a variety of plants species, these are indicated in the first column, accession number and second column, protein identity and source, thus the species that gave the highest identity score in the first (automated) search

Due to the unavailability of a *P. vittata* protein database, the identification of the obtained peptides was thus done using a cross-species search and alignment. Although some of the detected proteins were either hypothetical or predicted, some could be identified based on sequence identity with other plant proteins. To further authenticate each positively identified protein, the peptide sequence was used in a BLAST (Basic Local Alignment Search Tool) against the *Physcomitrella patens* database found in the Phytozome data base (<http://www.phytozome.net>). Phytozome is a joint project of the Department of Energy's Joint Genome Institute and the Center for Integrative Genomics to facilitate comparative genomic studies amongst green plants. *Physcomitrella* is a moss and is used as a model organism for studies on plant evolution, development and physiology. It has a lifecycle that includes a gametophyte and a sporophyte similar to that of ferns. It belongs to the Division of Bryophyta, seedless non-vascular plants, while ferns belong to the Division of Pterophyta, seedless vascular plants. Thus *Physcomitrella* is evolutionary close to ferns and the DataBase might be very useful

for protein identification. A phylogenetic tree that includes all plants in this DataBase is shown in Figure 26. A comparison of the phylogenetic position of the moss and fern is shown in Table XII.

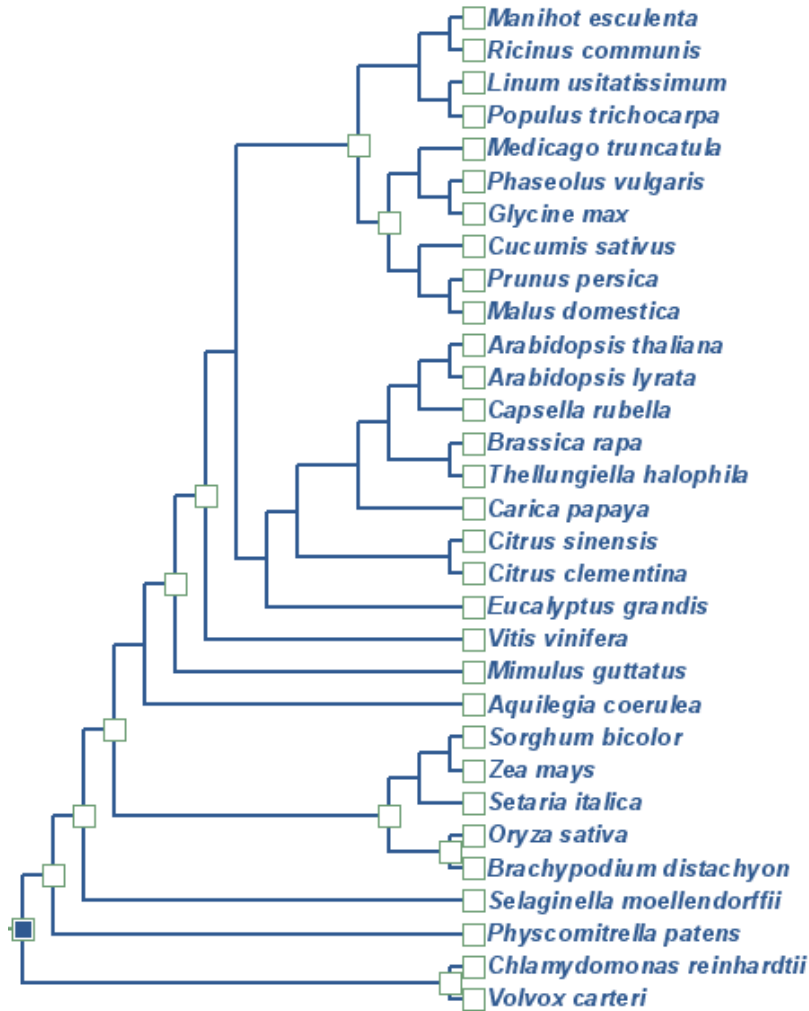


Figure 26: Phylogenetic tree of plants species in the Phytozome DataBase

For the reasons outlined above, an attempt was made to identify those proteins by using *Physcomitrella patens*. The results of the BLAST searches are in Table XI, at the right site and listed are the names of the proteins identified by the manual BLAST search against

Physcomitrella. In addition, the number of genes present in this organism is presented in the right hand column of Table XI.

Table XII: Systematics of *Pteris* and *Physcomitrella*

Pteris	Physcomitrella
Viridiplantae;	Viridiplantae;
Streptophyta;	Streptophyta;
Embryophyta;	Embryophyta;
Tracheophyta;	Bryophyta;
Moniliformopses;	Bryophytina;
Polypodiopsida;	Bryopsida;
Polypodiales;	Funariidae;
Pteridaceae;	Funariales;
<i>Pteris</i> .	Funariaceae;
	<i>Physcomitrella</i>

The identified proteins reveal that arsenic predominately affects proteins belonging to photosynthesis, carbon metabolism, protein turn-over and some proteins that are generally recognized as stress-related. They will be discussed in the next section.

6.3.3 Discussion of the Up-Regulated Proteins and Their Function.

The RPE gene encodes ribulose 5-phosphate 3-epimerase, EC 5.1.3.1 (spot 4) and catalyzes the reversible conversion of ribulose 5-phosphate and xylulose 5-phosphate in the pentose-phosphate pathway and Calvin cycle. The pentose-phosphate pathway is up-regulated under oxidative stress to provide NADPH for reduction of glutathione needed for removal of H₂O₂ (Liang et al., 2011). The dynamic rerouting of the metabolic flux of carbohydrates to the pentose-phosphate pathway has been established in yeast and *Caenorabditis* (Ralser et al. (2007).

The fructose biphosphate aldolase class 1 (spot 14 and 22) is an enzyme of the glycolytic pathway and gluconeogenesis as well as the pentose phosphate pathway, and catalyzes the reversible conversion of fructose 1,6 biphosphate into glyceraldehydes 3-phosphate and dihydroxyacetone 1-phosphate, EC 4.1.2.13. It is localized in the chloroplast. In *Arabidopsis* it was found to be involved in oxidative stress and Cd toxicity (Arabidopsis DataBase).

Enolase (spot 10, and 32), EC 4.2.1.11 appear to be up-regulated in Maize under anaerobic stress (Lal et al., 1998). Again this fits with the general change in carbon metabolism under stress conditions. Other proteins such as Glycosyl transferase group 1 ADP-glucose (spot26) fall in this category as well. They might be involved in damage repair of cell walls or energy metabolism.

As described in chapter 1, heavy metal accumulation can severely affect the photosynthetic pathway, reducing chlorophyll content, decrease in net photosynthesis and damage to the ultra-structure of chloroplasts. This was shown by Keiffer et al. (2008) in poplar plants under Cd stress. Down regulation of RuBisCO large and small subunit is a very common phenomenon resulted in affecting the photosynthetic pathway and was proven using various

plant species such as rice for Cd (Hajduch et al., 2001), Poplar for Cd (Keiffer et al., 2008), dwarf sunflower for As, see chapter 3. Since this analysis was not focused on down regulation of proteins under As stress, no such statement can be made about RuBisCO in *Pteris*. In addition, the precipitation with PEG removed the large subunit of RuBisCO. Spot 16 contained NADPH flavin oxidoreductase which is part of the photosystem. Up-regulation might be the result of damage to this system.

Protein spot 2, 27 and 31 were identified as chaperones and were up-regulated as well. Accumulation of high amounts of As in plant cells can damage proteins and enzymes which are involved in vital processes in the cells and chaperone activation is needed for proper protein folding, refolding, assembly, reassembly, degradation and translocation in the repair processes. Up-regulation of chaperones has been described in other proteomic studies dealing with response to metal stress including As. For instance, multifunctional chaperone 14-3-3 (spot 31) was identified in response to arsenate stress in *Chlamydomonas reinhardtii* which is discussed in chapter 5. The other two spots were chaperone cpn 60 which is abundant in mitochondria and plastids and is involved in the folding and oligomerization of newly synthesized polypeptides, assembly and disassembly of multimeric structures and protecting proteins from denaturation under different stress conditions (Nelson et al., 1992). It is possible that arsenic stress tolerance in *P. vittata* is in part the result of protection against protein denaturation or oxidation.

Spot 11 was identified as a peroxisomal membrane protein and is a good indication of antioxidative defense in *P. vittata*. Over-production of ROS has been demonstrated under biotic and abiotic stress and peroxisomes have a vital role in controlling the production of antioxidative enzymes such as SOD and other antioxidative enzymes involved in the glutathione-ascorbate cycle and the NADP-dehydrogenases located in this organelle (del-Río et al., 2006). This was

also reported in Pea leaves in response to Cd-induced stress (Romero-Puertas et al., 2002). Another protein up-regulated was ubiquitin thioesterase and was identified in spot 28 and 40. This is another indication of oxidative stress resulting in protein degradation. As previously explained, oxidative stress causes damage to intracellular proteins and proteolysis might be increased through the ubiquitin-proteasome pathway (Davies et al., 1988). Chen et al., (1994) have shown that the overexpression of the polyubiquitin gene in yeast helps prevent oxidative stress, suggesting that the ubiquitin-proteasome pathway might be important during oxidative stress. The proteolytic component of this pathway, also known as the 26S proteasome, is an enzymatic complex and involved in the degradation of short lived and abnormal cytosolic and nuclear proteins (Orlowski and Wilk, 2003). The same phenomenon was observed in *Chlamydomonas* in response to arsenate exposure as described in chapter 5.

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CHAPTER 7

OVERALL DISCUSSION

The long-term goal of this project was to elucidate the biochemical mechanism of toxic heavy metal accumulation and tolerance in plants. During this project the focus was mainly on arsenic (As) and lead (Pb) but with the project moving on, the emphasis has started to shift towards arsenic for the reasons outlined in chapter 1.

The Dwarf Sunflower *Helianthus annuus* (the cultivars Teddy Bear and Sundance) were used first because it is known to be a hyperaccumulator of Cd and As. Also Ni is accumulated in the presence of other metals (January et al., 2008). A proteomic approach was chosen to understand the molecular mechanism responsible for heavy metal hyperaccumulation because proteins are crucial to the vital functions in the cell. However, a proteomics approach is limited in its usefulness. There are two major disadvantages inherent to this method. First, despite the fact that proteins can be purified to a high state of purity and 2-D gel systems have become very sophisticated, it is impossible to analyze the entire proteome of an organism. Second, the proteomics approach relies on the premise that proteins are differentially expressed. Thus proteomics is only useful in detecting proteins that are up-regulated or down-regulated as the result of external stimulations. It is clear that other forms of control are possible, especially at the

enzyme level. Enzymes can be inhibited or activated without a change in their amount. On the other hand, if damage occurs to cellular structures such as multi-protein complexes or organelles, damage control might be by new synthesis to replace damaged molecules or by an increase in the removal process of damaged molecules and structures.

Thus, despite the limitation to a proteomics approach it was seen as a good first method to analyze events that take place during accumulation of metals. This was done by using one and two dimensional gel electrophoresis coupled with tandem mass spectrometry. Plant protein sample preparation for gel electrophoresis especially for 2-DE is critical due to several reasons explained in chapter 2. Therefore, the first task was to identify the best method for protein sample preparation. Altogether five methods were tested including conventional methods, but it was found that polyethylene glycol fractionation was the best approach for protein extraction from *H. annuus*. It gave the best resolution and maximum reduction of the amount of the large subunit of RuBisCO, which is highly abundant and obscures other proteins.

Since the major focus was to investigate the effects of arsenic, all the assays in which *H. annuus* was used included arsenic alone or in combination with other metals namely Cd, Cr and Ni. These were used since they are the major contaminant heavy metals found in Northeast Ohio and reflect the environment these plants will encounter when field experiments and later actual use in phytoremediation will begin. Some of the metal uptake experiments were performed at The University of Akron by Dr. Cutright and her students and it was shown that more metals were sequestered in roots than stems or leaves. The concentration values indicate that As uptake in *H. annuus* is not influenced by other metals, thus the process of accumulating arsenic in *H. annuus* appears to be governed by a distinct mechanism. When both cultivars were compared for metal uptake, the degree of metal accumulation in Teddy Bear differed slightly from that of

Sundance. The metal uptake in increasing order was Cr > Ni > As > Cd in Teddy Bear whereas the order in Sundance was Cr > Cd > Ni > As.

Expression of proteins in response to As was monitored by using a combination of Cd, Cr, Ni in the plants' growth medium with and without As and with As alone. Polyacrylamide gel electrophoresis revealed the presence of several extra proteins in the leaf extracts from Sundance treated with all four metals or with arsenic alone as compared to leaf extracts from control plants. Since these proteins were not apparent in either the control or leaf extracts from plants exposed to these three metals (Cd, Cr and Ni) alone, it was concluded that the expression of these proteins was due to the effect of arsenic. Among the polypeptides discovered one with an approximate molecular mass of 32 kDa was very strongly induced, even visible on 1-D gels and was, therefore, chosen for further analysis. This polypeptide was identified as chitinase using LC-MS/MS. The gene was cloned and its nucleotide and amino acid sequence determined. It is the first Dwarf Sunflower gene cloned and sequenced.

The up-regulation of chitinase in response to As exposure was proven by semi-quantitative RT-PCR with RNA isolated from leaves of control plants, and from plants exposed to Cd alone and from plants exposed to all metals with or without As. The chitinase mRNA was detected only when plants were exposed to As.

Final proof that indeed this protein is chitinase was provided by Western blot analysis with an antibody generated against chitinase. It clearly showed the presence of a 32 kDa protein in both Sundance and Teddy Bear samples, and was specifically up-regulated when As was added to the plants, alone or in combination with other metals. Apparently Sunflowers express this enzyme under conditions induced by exposure to As. What the actual function of this protein is, remains unclear. Its normal role is protection against fungal infection. Thus, the major

conclusion from this work is that exposure of the plant to As is a condition of stress. Apparently both biotic and abiotic stress results in the induction of the chitinase gene. Its function is unclear in this respect: is it a general response as the result of cell death and can it be seen as a general, unspecific, form of defense?

With the availability of specific antiserum against chitinase, the presence and location of this enzyme can now be followed in roots and stems. This was tested using dot-blot analysis with leaf, stem and root proteins. In the presence of As chitinase is expressed strongly in the roots of soil-grown plants while leaves and stems do not show any expression. The hydroponically-grown plants show a much higher response to As in the leaves. This suggests that entry and transport of As is much better in hydroponically grown plants. The results also show that detection of chitinase is a good method to see stress in the plant. It even might be a good method to detect As (or Pb) presence in soils when Sunflowers are grown on a soil with unknown chemical contamination.

Expression in *H. annuus* of proteins in response to Pb exposure alone or with the other three metals was investigated next. Six proteins were detected in preliminary one-dimensional gel electrophoresis and among them were ATP synthase α and β subunits, malate dehydrogenase (MDH), carbonic anhydrase, RuBisCO activase, triose phosphate isomerase and nitrophenyl phosphatase (phosphoglycolate phosphatase). These enzymes were predominant, which was described in chapter 4. From the obtained results the picture is emerging that toxic metals such as Pb and As cause a general defense mechanism to be turned on, which is exemplified by chitinase. In addition, photosynthesis is impaired which results in up-regulation of the synthesis of enzymes in the carbon flux to offset a diminished level of photosynthesis due to damage to photosystems and degradation of RuBisCO. Degradation of RuBisCO was shown on 1-D gels

before methods were developed to remove it specifically for 2-D gels. The degradation of RuBisCO must have a major effect on metabolism and the up-regulation of the activase might be to compensate for the loss of RuBisCO to stimulate the remaining enzyme. What is striking is that another enzyme that was up-regulated was carbonic anhydrase which catalyzes the formation of CO₂ gas from the soluble bicarbonate and so increases the concentration of accessible, substrate, CO₂ for RuBisCO. Because RuBisCO also catalyzes the photorespiration of ribulose 1,5 bisphosphate to 3-phosphoglycerate and 2-phosphoglycolate, which is normally a loss of carbons for sugar and energy production, the phosphoglycolate phosphatase was found to be up-regulated to increase the recycling of carbon and, as shown in chapter 4, to become glycolate, which is converted to glyoxalate and further used in a recovery pathway that will recover $\frac{3}{4}$ of the carbons.. This notion is supported by the fact that MDH which is one of the enzymes involved in the regeneration of glyoxylate was up-regulated as well. The RuBisCO activase requires ATP, this is probably also the reason that ATP synthase subunits were found to be up-regulated.

Thus, some enzymes in critical pathways are expressed at a higher level to compensate for loss in the carbon flux. That not all enzymes of these pathways were found could be because proteomics is not sensitive enough to detect smaller differences in amounts and these enzymes are simply they are below the detection level. It also is possible that some of these enzymes are regulated in a different way: the amounts stay constant but their activities are modified. Clearly a physiological and biochemical approach will be needed to prove these points. However, the observation that damage and damage-repair takes place is a clear result of this proteomics approach. The work with the Ferns also has shown effects on metabolic pathways; in this case it appears that the pentose phosphate pathway is up-regulated to produce reducing power to offset

oxidative damage. It is tempting to speculate that this might be needed to reduce arsenate to arsenite, which is transported by the arsenite transporter to the vacuole. This appeared to be the evolutionary mechanism for this Fern to grow and prosper on arsenate-contaminated soils.

Oxidative damage is a recurring theme and was discussed in chapters 3 and 4 and in the literature cited in these chapters. However, with both Sunflower and Fern the added complexity to this investigation was twofold. One, there are no genomic DataBases for both plants, although there is a Sunflower Gene Index, which consists of a collection of sequenced mRNAs and is not comprehensive. Analysis of mass spectrometry results has to be an indirect approach, first a search against known plant DataBases and then a manual recheck against the Gene Index sequences. Secondly, both plant species are complex organisms with many different tissues and the response that was observed could be more than just a response at the cellular level.

Therefore, it was decided to investigate whether a unicellular green alga, *Chlamydomonas reinhardtii*, would be a good model organism to study the effect of As. This investigation was begun with basic cell growth analysis and it was found that *Chlamydomonas* was unable to sustain growth in the presence of arsenate at doses above 100 μM , thus it is incapable of detoxification of arsenate. A proteomic analysis was performed next. However, standard methods as used before were not suitable for extraction of *Chlamydomonas* proteins. It was found that ammonium sulfate fractionation was the best method, because it removed contaminants and was able to recover more than 90% of the original proteins from the crude extract: it simply removed nucleic acids and RuBisCO. Fifteen proteins were over-expressed when the cells were treated with 200 μM arsenate and most of them are associated with the removal of damaged proteins, oxidative stress, increased energy demand, protein synthesis and protein folding. The majority of the cell's reaction appears mainly to be a response to protein and

oxidative damage which is slightly different from what was observed for Sunflowers. However, the similarity to Sunflowers is seen in the increase of some metabolic enzymes, which suggests that photosynthesis and sugar metabolism is impaired. Other enzymes might be up-regulated as well but again might be below the detection limit of the protein identification. No specific As-binding proteins were found as was one of the premises of this project.

As a final conclusion it can be stated that it appears that *C. reinhardtii* might not be a good model to study the response to As uptake, but it confirmed the observation that protein damage, reactive oxygen species and general carbon metabolism are targeted by arsenic poisoning and that plants respond to this at the cellular level.

Thus, Sunflowers can accumulate As because they have a general defense mechanism plus they deal with oxidative stress. In part this might be by isolating the As, but that has not been proven. Mainly they deal with it by repair and removal of damaged cells. Ferns hyperaccumulate As as an evolutionary mechanism to protect themselves. There is no oxidative stress because they have an anti-oxidative mechanism in place and remove the toxin quickly. From a viewpoint of optimal phytoremediation in Ohio, Sunflowers are the best “tools” because the Ferns do not grow in a cool climate. Maybe genetic engineering of the Fern arsenite transporter into Sunflowers would be the solution to remedy As-poisoned soils in Northeast Ohio.