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CHARACTERIZATION OF OXIDATIVE STRESS RESPONSE PROTEINS IN METAL-TOLERANT AND METAL-SENSITIVE *BRASSICACEAE* PLANT SPECIES

A Thesis

by

LUIS M. DE SANTIAGO

Submitted to the Graduate School of
The University of Texas Pan-American
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2015

Major Subject: Biology

CHARACTERIZATION OF OXIDATIVE STRESS RESPONSE PROTEINS IN METAL-TOLERANT AND METAL-SENSITIVE *BRASSICACEAE* PLANT SPECIES

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August 2015

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ABSTRACT

De Santiago, Luis M., Characterization of Oxidative Stress Response Proteins in Metal-Tolerant and Metal-Sensitive Brassicaceae Plant Species. Master of Science (MS), August, 2015, 49 pp., 13 figures, 47 references, 26 titles.

In this study the expression of oxidative stress response (OSR) proteins in nonaccumulator and hyperaccumulator plant species of the *Brassicaceae* family were characterized. Oxidative stress was induced via nickel treatment and proteins were characterized through western blotting and image analysis. Statistical analysis indicated that the nonaccumulator species *Arabidopsis thaliana* significantly decreased catalase (CAT), glutathione reductase (GR), copper/zinc-dependent superoxide dismutase (Cu/ZnSOD), iron-dependent SOD (FeSOD), manganese-dependent SOD (MnSOD) and metal tolerance protein 1 (MPT1) expression in response to Ni-treatment. The hyperaccumulator species *Thlaspi montanum* variety *montanum* displayed either no significant change or a significant increase in OSR regulation following Ni-treatment with the exception of CAT. Overall, the nonaccumulator group displayed a significant decrease in total OSR regulation following Ni-treatment as compared to the other groups. These results suggest that the sustained expression of OSR proteins during oxidative stress may be one of the mechanisms that influence metal tolerance in hyperaccumulator plant species.

DEDICATION

To my parents, for demonstrating that there is nothing that can hold you back regardless of your circumstances. For my sister, for being the foundation of my family and allowing me to pursue my ambitions and dreams. Without her, the completion of this thesis and the aspirations that followed would not have been possible. And finally, to my friends since childhood who have never failed to provide me with spirit.

ACKNOWLEDGEMENTS

I would like to acknowledge my committee members Dr. Michael Persans, Dr. Megan Keniry, and Dr. Robert Gilkerson as well as former member Dr. Robert Dearth for providing me with the avenues that made the completion of this thesis possible. I would like to personally thank Dr. Michael Persans for providing me with the opportunity to pursue graduate research in his laboratory. The simple decision of providing me with his guidance, time, and resources has immeasurably affected my life for the best.

I would like to thank my past and present laboratory research companions Madeleine Sano, Selena Urbina, Thalia Rios, Yessica Cerino, and Elyssa Garza for not only helping me with my research, but for providing me with laughter and memories that can never be replicated. I would also like to thank my classmates who have helped me navigate my graduate career and personal life. Their friendship and support has made this an unforgettable journey. A special thanks goes to Dr. Alexis Racelis for making a career in ecological and agricultural research not only a tangible prospect, but a fulfilling and rewarding investment worth pursuing. I would also like to thank Thomas Eubanks for providing me and the laboratory with not only his engineering expertise, but with his light-hearted charisma. Special thanks goes to Dr. Christopher Vitek and Dr. Fred Zaidan for showing me that you can be a fun, enthusiastic, and inspiring individual in the field of science.

Lastly, I would like to thank the sponsorship provided by the USDA HSI program for providing me with the financial resources required for this endeavor.

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CHAPTER I

INTRODUCTION

Reactive oxygen species (ROS) are endogenously produced in plants through aerobic metabolism and photosynthesis. These ROS are capable of damaging cellular structures such as nucleic acids, proteins, and lipids. ROS can also produce additional amounts of free radicals through subsequent oxidation reactions which results in further cellular damage (Sies 1997). A steady-state concentration of ROS is maintained in the cells at sub-lethal levels by enzymatic and non-enzymatic antioxidants. Oxidative stress response (OSR) proteins within a cell under normal environmental conditions contribute to the reduction of ROS. Elevated production of ROS can overwhelm a cell's antioxidant defense mechanisms, which results in increase oxidative stress. Oxidative stress in turn can impair cellular function which adversely affects the health of the plant (Foyer et al. 1997).

Exogenous ROS and other free radicals can also be produced by transition metals such as Cu, Fe, Ni, Zn and Cd. These transition metals mediate redox reactions with oxygen and water resulting in the production of free radicals such as superoxide ($O^{\cdot -}_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) (Sies 1997). The unmanaged ROS can result in DNA oxidation, lipid peroxidation, and protein oxidation which can ultimately result in impaired DNA replication, membrane damage, and inhibited protein function (Ercal et al. 2001).

Transition metals can also compromise OSR proteins in two different manners; they can bind to sulfhydryl groups which can disrupt their catalytic activity and the structural integrity of

the protein (Babaoğlu et al. 2009). They may also displace the metallic cofactor of metalloproteins reducing their efficiency and effectiveness (Dudev and Lim 2001). Increased ROS due to metal accumulation can result in metal-induced oxidative stress which is detrimental to the plant.

Plants are capable of tolerating steady state ROS levels, in part, due to enzymatic OSR proteins such as superoxide dismutase, catalase and glutathione reductase. This antioxidant defense mechanism scavenges free radicals which allow plants to tolerate growth in soils that contain sub-lethal levels of metal concentrations. However, there are a group of plants that display the ability to tolerate growth in metalliferous soils and actively accumulate metals within their plant tissue at concentration levels that are higher than those found in the soil. These metal-tolerant, hyperaccumulators exhibit the overexpression of genes responsible for transmembrane transporters such as those found in the ZIP, HMA, MTP, and YSL families (Guerinot 2000; Gendre et al. 2007; Adams et al. 2011; Ricachenevsky et al. 2013). These genes are responsible for encoding proteins that translocate metal ions throughout the plant (Rascio and Navari-Izzo 2011) and allow them to accumulate more than 1,000 μg g^{-1} of nickel, and 10,000 μg g^{-1} of zinc or manganese (Baker and Brooks 1989).

Metal-tolerant, hyperaccumulators can tolerate high levels of metals within their tissues without succumbing to metal-induced, oxidative stress. This phenotype is displayed in over 450 plant species, many of which belong to the *Brassicaceae* family. Hyperaccumulator plants are capable of accumulating and tolerating increased concentrations of metals within their tissues, but these two phenotypes are genetically independent traits; the aforementioned metal transporter gene families are responsible for mediating the increased movement of metals throughout the plant but are not directly responsible for tolerating the increased ROS produced by the

accumulated metal (Maestri et al. 2010). Two possible mechanisms employed by hyperaccumulators are to reduce the amounts of toxic metals in the cytosol with the transporter proteins and to increase the expression of OSR genes in order to tolerate the increased amount of ROS generated by metal accumulation.

Previous research indicates that the proteins dihydrolipoamide dehydrogenase (LADH), glutathione S-transferase (GST), and a mannose-6-phosphate reductase (an oxidoreductase) which are implicated in oxidative defense are induced in the shoot biomass of *Alyssum lesbiacum* when exposed to 0.3 mM $NiSO_4$ (Ingle et al. 2005). Similarly, iron-dependent superoxide dismutase (Fe-SOD) and glyoxalase I (GLXI) are also induced in *Arabidopsis halleri* when exposed to 1.0 mM $CdSO_4$ and 10 mM $ZnSO_4$ (Farinati et al. 2009). An increase in the OSR gene expressions of *catalase* (*CAT*), *superoxide dismutase* variants (*SOD*), *glutathione reductase* (*GR*), and *metal tolerance protein 1* (*MTP1)* may be similarly occurring in other hyperaccumulator species.

Hydrogen peroxide will oxidize ferrous iron ($Fe2^{+}$) into the ferric iron (Fe^{3+}) which results in the formation of a hydroxyl radical ($OH^{\cdot}$) and hydroxide anion (OH^{-}). This reaction, known as the Fenton reaction, occurs when hydrogen peroxide is in the presence of iron and results in potentially damaging ROS (Mhamdi et al. 2010). Catalase (CAT) is responsible for the decomposition of hydrogen peroxide into atmospheric oxygen and water.

Glutathione reductase (GR) or, glutathione-disulfide reductase (GSR) is responsible for catalyzing the reduction of glutathione disulfide (GSSG) to glutathione (GSH). GR sustains the sulfhydryl form of GSH which is a critical non-enzymatic antioxidant responsible for reducing the disulfide bonds between proteins such as those in the amino acid cysteine to form its derivative cystine (Dumas 2012). GSH is able to donate a reducing equivalent from cysteine to a

ROS stabilizing the molecule. GSH becomes reactive in the process and will bond with another GSH forming GSSG which is then reduced by GR. GR also plays a role in the decomposition of hydrogen peroxide in the glutathione-ascorbate cycle by maintaining the reduced state of GSH which reduces dehydroascorbate (Gill et al. 2013).

Zinc is an essential micronutrient responsible for the activity of over 300 enzymes by serving a catalytic function or providing protein structure and stability (McCall et al. 2000). The metal tolerance protein 1 (MTP1) is a member of the cation diffusion facilitator family responsible for the sequestration of Zn into the vacuoles of leaf epidermal cells (Kobae et al. 2004) and is believed to provide a mechanism in conferring metal tolerance in hyperaccumulator plant species such as those of the genus *Thlaspi* (Küpper et al. 1999). The zinc-resistance-conferring (*ZRC1*) gene similar to *MTP1* has been demonstrated to confer oxidative stress resistance in the yeast *Saccharomyces cerevisiae* (Kobayashi et al. 1996). This further suggests that the allocation of Zn by MTP1 may be a mechanism for hyperaccumulator plants for tolerating oxidative stress.

Superoxide dismutase (SOD) is responsible for the disproportionation of the superoxide radical ($O^{\cdot-}_2$) into atmospheric oxygen or hydrogen peroxide; SOD can either add or remove an electron allowing it to alternatively generate both products. SOD requires a metallic cofactor to function and can be classified into four groups: iron SOD (FeSOD), manganese SOD (MnSOD), copper-zinc SOD (Cu/ZnSOD), and nickel SOD (NiSOD) (Abreu and Cabelli 2010).

Zinc is also metallic cofactor for Cu/ZnSOD and is bound by two histidines, an asparate, and a bridging imidazole (Abreu and Cabelli 2010). Zinc provides structural integrity to Cu/ZnSOD while the copper ion (Cu^{2+}) provides the catalytic chemistry (Hough and Hasnain

2003). The sequestration of Zn by MTP1 may influence the availability of Zn as a cofactor which can potentially influence the activity of Cu/ZnSOD.

The characterization of expression levels of the OSR proteins CAT, GR, MTP1, Cu/ZnSOD, FeSOD, and MnSOD in hyperaccumulator plant species can provide insight into how hyperaccumulator plants tolerate large concentrations of metals as opposed to their non-accumulator plant counterparts. The roles that the OSR proteins CAT, GR, MTP1, Cu/ZnSOD, FeSOD, and MnSOD perform in the management of oxidative stress are important to understand in order to develop a more efficient and effective hyperaccumulator plant for the purpose of phytoremediation.

Statement of the Problem

Waste products generated by agriculture, industrial, and urban activities result in the contamination of soils with high amounts of heavy metals such as cadmium (Cd), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn) (Naveedullah et al. 2013). Unlike organic contaminants which can be degraded or metabolized by microflora and plants (Reichenauer and Germida 2008), metal contaminants accumulate in soils and can potentially be leached into neighboring water systems where they enter the food chain (Khan et al. 2008). Increased human exposure to heavy metals such as cadmium and lead can lead to renal, liver, and bone damage (Godt et al. 2006) as well as nervous, hematopoietic, endocrine, and reproductive system damage (Pirkle et al. 1998), respectively. Metal pollutants from municipal waste, agrochemicals, and industrial runoff (Islam and Tanaka 2004) also cause damage to estuarine, riverine, and marine ecosystems where they bioaccumulate (Hsu et al. 2011).

Metal contaminated soils can be remediated through multiple means. Contaminated soils can be removed from site, transported to a facility for chemical and physical treatment, and then returned to its original site (Ghosh and Singh 2005). This *ex situ* method results in disruption of natural biological processes that occur in the soil and inevitably damages the physio-chemical characteristics that allows for the productivity of soil (Gliessman 2007). Metal polluted soils can also be treated *in situ* through the application of organic and inorganic amendments in order to immobilize metals and decrease their bioavailability (Abumaizar and Smith 1999; O'Dell et al. 2007). Although *in situ* treatments of metal-contaminated soils are less disruptive they do not result in the removal of metals from the soil and require continuous administration in order to keep the metals inert.

Plants capable of hyperaccumulating large amounts of metals provide an environmentally friendly solution for remediating contaminated soils without damaging soil fertility and structure (Ghosh and Singh 2005). Phytoremediation, through phytoextraction, is the utilization of plants for absorption and accumulation of contaminants from soils and water into plant biomass. The accumulated metal contaminant can then be retrieved and recycled for profit in a process known as phytomining (Anderson et al. 1999).

Hyperaccumulators must be able to rapidly extract large amounts of metals from the soil in order to make phytoremediation a practical alternative. Unfortunately, most hyperaccumulators are slow growing, develop a shallow root system, and have a small biomass. The efficacy of phytoextraction is thus limited by these innate features. Biotechnology may provide a possible solution to this limitation by allowing the transgenic engineering of a hyperaccumulator plant with the ideal characteristics for phytoextraction. The mechanisms by which a hyperaccumulator tolerates metal and manages ROS must be better understood in order

to engineer the ideal hyperaccumulator plant. The characterization of OSR protein expression in oxidative stress management between nonaccumulator and hyperaccumulator plant species may reveal a mechanism for the hyperaccumulation and tolerance of heavy metal.

Conceptual Framework

Expression of oxidative stress response (OSR) genes *CAT, GR, MTP1, Cu/ZnSOD FeSOD,* and *MnSOD* were characterized from 100 µM nickel acetate-treated and non-treated (control) *Brassicaceae* plant species. Total protein was extracted from the nonaccumulator plant species *Arabidopsis thaliana*, *Brassica juncea*, and the metal-tolerant species *Streptanthus farnsworthianus* and hyperaccumulator plant species *Streptanthus polygaloides, Thlaspi montanum* var. *montanum* and *Thlaspi montanum* var. *siskiyouense*. Concentration of protein in the samples was determined by Bradford protein assay and detection of proteins of interest was conducted via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by an immunoblot (western blot) with selected antibodies. Quantification of protein expression was conducted by measuring the pixel density (of optical scans) of bands on western blots via the image processing software ImageJ (Abràmoff et al. 2004).

The goal of this study was to determine if there are significant differences in the expression of OSR genes between nonaccumulator and hyperaccumulator plant species and to determine if increasing OSR expression is a mechanism employed by hyperaccumulator plants to tolerate heavy metals. The results may then be used to aid in the engineering of an ideal hyperaccumulator plant for the purposes of phytoremediation of metal-contaminated soils through phytoextraction.

Statement of Purpose

The purpose of this thesis was to induce oxidative stress via nickel treatment in a set of nonaccumulator and hyperaccumulator plant species in order to characterize the protein expression of the OSR genes *CAT*, *GR*, *MTP1 Cu*/*ZnSOD*, *FeSOD*, and *MnSOD*. The hypothesis in this study is that hyperaccumulator plant species over-express these OSR genes producing higher levels of OSR proteins in order to manage the increased production of ROS as compared to the nonaccumulator plants. Understanding the mechanisms employed by hyperaccumulators for the management of metal-induced oxidative stress may reveal specific genes used by *Brassicaceae* plants for metal tolerance. These genes can then be utilized to engineer an ideal hyperaccumulator plant for the phytoremediation of metal contaminated soils through phytoextraction.

CHAPTER II

MATERIALS AND METHODS

Selected *Brassicaceae* Plant Species for Study

Plant species were selected from the *Brassicaceae* family based on their ability to accumulate heavy metal. The three nonaccumulator species used were *Arabidopsis thaliana* var. *columbia*, *Brassica juncea*, and the metal-tolerant species *Streptanthus farnsworthianus*. The three hyperaccumulator species were *Streptanthus polygaloides*, *Thlaspi montanum* var. *montanum*, and *Thlaspi montanum* var. *siskiyouense*.

The seeds used for the nonaccumulator plant species *A. thaliana*, *B. juncea*, and *S. farnsworthianus* were provided by LEHLE seeds (Round Rock, TX), the United States Department of Agriculture (USDA) seed bank, and by Ron Ratko of Northwest Native Seeds (Seattle, WA), respectively. The seeds used for the hyperaccumulator plant species *S. polygaloides* were provided by Robert Boyd of Auburn University (Auburn, AL), while the seeds for the hyperaccumulator plant species *T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense* were both provided by Ron Ratko of Northwest Native seeds (Seattle, WA).

Plant Growth and Treatment

Plants were previously grown in hydroponic tubs containing 8 liters of 0.1 X Hoaglands nutrient solution consisting of 200 mL of 4 X Hoaglands solution, 40 mL of 40 mM FeDTPA, and 7.76 liters of distilled water as described by Into (2010) under environmentally controlled

conditions. Plants were treated with 2 mL of 400 mM Nickel Acetate producing a 100 µM Ni concentration in the hydroponics solution for 12 days after 2 to 3 months of plant growth. In hydroponics solution without Nickel, a second, control set of plants did not receive treatment. The plants were collected after 12 days, flash frozen in liquid nitrogen, ground to a fine powder via sterile mortar and pestle, and stored at -80°C.

Protein Isolation from Plant Tissue

Three hundred and fifty µL of plant tissue was frozen with liquid nitrogen, ground to a fine powder in a sterile mortar and pestle, and transferred to a 1.5 mL test tube. Protein isolation buffer containing 0.1 M Tris, 0.5 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 20 mM sodium ascorbate, 1 mM dithiothreitol, 1.25 mg/mL insoluble polyvinylpolypyrrolidone (PVP), 0.1 M phenyl methyl sulfonyl fluoride (PMSF), and 33 µL of 5 µg/mL protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) was added to the test tube containing powdered plant tissue. The test tube was kept in ice and the sample was ground for 30 seconds with a VWR™ pellet and motor mixer (VWR International, Radnor, PA). The test tube was centrifuged at 14,000 RCF, at 4°C, for 15 minutes. Supernatant was transferred to a new 1.5 mL test tube and centrifuged a second time at 14,000 RCF, at 4°C, for 15 minutes. The supernatant was transferred to a new 1.5 mL test tube and stored at -20°C.

Quantification of Protein Concentration

The concentration of protein in a sample was measured using a Bradford protein assay kit (Bradford, 1976) from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). Twenty microliters of protein sample was transferred to a 1.5 mL cuvette containing 800 µL of sterile water and 200

µL of BioRad protein assay reagent and stood at room temperature for 5 minutes. A control sample was prepared by substituting the 20 µL of protein sample with 20 µL of isolation buffer. Spectroscopic analysis was conducted with a BioRad SmartSpec3000 spectrophotometer and absorbance of samples was measured at 595 nm (BioRad, Hercules, CA). The concentration (mg/mL) of protein was then derived from the equation of the standard protein curve: (A_{595} + 0.0106 / 0.5472).

Western Blotting and Imaging Analysis

SDS-PAGE and Electroblotting

Twenty µg of protein sample per 12.5 µL of isolation buffer, and 12.5 µL of protein loading buffer containing 0.1 M Tris base, 4% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 10% glycerol, and 1 mg/mL bromophenol blue (pH 6.8) were transferred to a 1.5 mL test tube. Test tubes were placed in a boiling water bath for 5 minutes then allowed to air dry in the laminar flow cabinet for 20 minutes. The test tubes were then centrifuged at 14,000 RPM for 5 minutes. Twenty five microliter samples were loaded into individual wells of 12% Mini-Protean® TGX, 10 well, 30 µL capacity, precast polyacrylamide gels (BioRad, Hercules, CA). A Spectra™ Multicolor Broad Range Protein Ladder (SMBR-PL) (Thermo Fisher Scientific, Waltham, MA) was loaded into the first well of the gel and a blank solution containing only 12.5 µL of isolation buffer and 12.5 µL of protein loading buffer was loaded into the last well. The SDS-PAGE apparatus was ran at a constant 150 volts for 75 minutes.

Transfer stacks consisting of 2 Extra Thick Blot Paper (BioRad, Hercules, CA) filter papers and 1 PVDF membrane (Micron Separations Inc., Westborough, MA) were prepared by soaking the filter papers for 10 minutes in transfer blotting buffer containing 48 mM Tris, 39

mM glycine, 0.04% SDS, and 20% methanol and by soaking the PDVF membranes in 100% methanol for 10 seconds. Precast gels were opened using a Mini-Protean® Cassette Opening lever and the gel membranes were removed and placed into the transfer stacks in the following arrangement from bottom to top: filter paper, PVDF membrane, gel membrane, and filter paper. The transfer stacks were allowed to equilibrate in the transfer blotting buffer for 10 minutes then were individually electroblotted using a Trans-Blot® SD Semi-Dry Transfer cell (BioRad, Hercules, CA) at a constant 14 volts for 45 minutes. The PVDF membranes were removed from the transfer stack and blocked with 5% Carnation Instant Non-Fat Dry Milk (Nestlé, Vevey, Switzerland) over night at room temperature. The 5% non-fat milk was decanted and PVDF membrane was washed with 20 mL of TBST consisting of 0.5 M Tris, 1.5 M sodium chloride, and 0.5% Tween-20 (pH 8.0 with hydrochloric acid) for 10 minutes. TBST wash was repeated 2 more times for a total of 3 washes for 30 minutes each.

Detection of Actin

PVDF membrane was incubated with 10 µL of 1 mg/mL of actin-mouse primary antibody (Antibodies-Online Inc., Atlanta, GA) diluted in 10 mL of TBST buffer consisting of 0.1% gelatin (1:1,000 dilution) for 1 hour and 30 minutes at room temperature on top of a rocker. Primary antibody was then removed and the PVDF membrane was washed 3 consecutive times, 10 minutes each with 20 mL of TBST buffer. The PVDF membrane was then incubated with 1 µL of 0.3mg/mL Pierce® Rabbit Anti-Mouse IgG, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) diluted in 10 mL of TBST buffer consisting of 0.1% gelatin (1:10,000 dilution) for 1 hour at room temperature. The secondary

antibody was then removed and the PVDF membrane was washed 3 consecutive times, 10 minutes each with 20 mL of TBST buffer.

Detection with Other Antibodies

Detection of other antibodies was performed as per detection of actin with the following changes: 10 µL of catalase-rabbit primary antibody (Agrisera, Vännäs, Sweden) and 1 µL of 0.3mg/mL Pierce® Goat Anti-Rabbit IgG, (H+L), and 1 µL of 0.3mg/mL Pierce® Goat Anti-Rabbit IgG, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) Phosphatase conjugated secondary antibody for CAT detection.

Five micro liters of 7 mg/mL glutathione reductase-rabbit primary antibody (Agrisera, Vännäs, Sweden) diluted in 25 mL of TBST buffer consisting of 0.1% gelatin (1:5,000 dilution and 1 µL of 0.3mg/mL Pierce® Goat Anti-Rabbit IgG, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) for GR detection.

Ten micro liters of MTP1-rabbit primary antibody (Agrisera, Vännäs, Sweden) and with 1 µL of 1mg/mL Pierce® Goat Anti-Rabbit IgG, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) for MTP1 detection.

Ten micro liters of 2.3 mg/mL Cu/Zn-dependent superoxide dismutase-hen primary antibody (Agrisera, Vännäs, Sweden) and 1 µL of 1mg/mL Pierce® Goat Anti-Chicken IgY, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) for Cu/ZnSOD detection.

Five micro liters of iron-superoxide dismutase-rabbit primary antibody (Agrisera, Vännäs, Sweden) diluted in 25 mL of TBST buffers consisting of 0.1% gelatin (1:5,000 dilution)

and 1 µL of 0.3mg/mL Pierce® Goat Anti-Rabbit IgG, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) for FeSOD detection.

Five micro liters of manganese-dependent superoxide dismutase-rabbit primary antibody (Agrisera, Vännäs, Sweden) diluted in 25 mL of TBST buffers consisting of 0.1% gelatin (1:5,000 dilution) and 1 µL of 0.3mg/mL Pierce® Goat Anti-Rabbit IgG, (H+L), Alkaline Phosphatase conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA) for MnSOD detection.

Colorimetric Detection

The PVDF membranes were incubated with 32.5 µL of BCIP and 65 µL of NBT color development substrates (Promega, Fitchburg, WI) at room temperature in 10 mL of alkaline phosphatase buffer consisting of 100 mM Tris, 100 mM sodium chloride, 5 mM magnesium chloride (pH 9.5) for 25 minutes under dark conditions. The PVDF membranes were then allowed to air-dry overnight under dark conditions.

Analysis of Membrane Band Densities

Quantification of protein expression was performed by scanning the PVDF membrane with a Hewlett Packard Scanjet 3570c (Hewlett Packard, Palo Alto, CA) scanner using 1200 DPI, greyscale, TIFF output settings. The band densities on the TIFF image were analyzed via the image processing software ImageJ (Rasband, ImageJ, NIH). TIFF image was horizontally aligned using the "rotate" function in order to accurately select bands and were converted to a profile plot using the "plot lanes" function. Background signal, or noise, was manually removed

using the “straight line” and “wand” functions and the remaining signal was expressed as the pixilated area, or density, of the band. The resulting values were used for statistical analysis.

Imaging and Statistical Analysis

CAT, GR, MTP1,Cu/ZnSOD, FeSOD, and MnSOD (OSR) band densities generated by Western Blotting for both control and 100 µM nickel-treated plants were quantified via ImageJ.

Ni-treated averages for ACT expression per species were divided by their pair control averages to establish an expression constant then ACT control values per species were normalized to 1. All OSR Ni-treated values were divided by their respective expression constant then divided by their pair control values. All OSR control values were then normalized to 1.

Independent student t-tests were performed between control and Ni-treated expression values per plant species using standard deviations from non-transformed control values. One- and two-way analysis of variance (ANOVA) and post hoc Tukey tests were conducted via the statistical software JMP® (SAS Institute Inc., Cary, NC) using transformed data in order to satisfy normal distribution assumption.

CHAPTER III

RESULTS

OSR Protein Expression per Species

Catalase Expression

Twelve days of Ni exposure resulted in a significant difference in CAT protein amounts ($P<0.05$) between 100 µM Ni-treated plants versus control plants for the non-hyperaccumulator species *A. thaliana*, *B. juncea*, and *S. farnsworthianus* as well as for the hyperaccumulator species *T. montanum* var. *montanum*, *T. montanum* var. *siskiyouense*, and *S. polygaloides*: ($p < 0.0001$, $p < 0.0001$, $p < 0.01$, $p < 0.05$, $p < 0.0001$, $p < 0.01$); respectively (Figure 7). There was a 0.51 fold decrease in *A. thaliana*, 0.37 fold decrease in *B. juncea*, and a 0.66 fold decrease in *S. farnsworthianus* 100 µM Ni-treated, CAT bands as compared to the control bands (Figure 1). There was also a 0.49 fold decrease in *S. polygaloides*, 0.52 fold decrease in *T. montanum* var. *montanum*, and a 0.12 fold decrease in *T. montanum* var. *siskiyouense* 100 µM Ni-treated, plants as compared to the control bands (Figure 1).

Glutathione Reductase Expression

Twelve days of Ni exposure resulted in a significant difference ($P<0.05$) between the 100 µM Ni-treated plants versus the control plants of the non-hyperaccumulator species *A. thaliana*, *B. juncea* and metal-tolerant *S. farnsworthianus* as well as the hyperaccumulator species *S. polygaloides*: ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.001$); respectively (Figure 8). There was no

significant difference measured between the 100µM Ni-treated plants and control plants of the hyperaccumulator species *T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense*: (p = 0.15, p = 0.09); respectively (Figure 8). There was a 0.72 fold decrease in *A. thaliana*, a 0.61 fold decrease in *B. juncea*, a 0.71 fold decrease in *S. farnsworthianus* as well as a 0.62 fold decrease in *S. polygaloide*s 100 µM Ni-treated, GR bands as compared to the control bands (Figure 2).

MTP1 Expression

Twelve days of Ni exposure resulted in a significant difference ($P < 0.05$) between the 100 µM Ni-treated plants versus the control plants for the non-hyperaccumulator *A. thaliana*, and *B. juncea* as well as for the hyperaccumulator *T. montanum* var. *siskiyouense*: ($p < 0.0001$, $p < 0.01$, $p < 0.0001$); respectively (Figure 9). There was no significant difference measured between the 100 µM Ni-treated plants versus the control plants for the metal-tolerant species *S. farnsworthianus* and the hyperaccumulator species *S. polygaloides* and *T. montanum* var. *montanum*: ($p = 0.23$, $p = 0.08$, $p = 0.06$); respectively (Figure 9). There was a 0.36 fold decrease in *A. thaliana*, a 0.58 fold decrease in *B. juncea*, as well as a 0.18 fold decrease in *T. montanum* var. *siskiyouense* 100 µM Ni-treated, MTP1 bands as compared to the control bands (Figure 3).

Copper/Zinc-Dependent Superoxide Dismutase Expression

Twelve days of Ni exposure resulted in a significant difference ($P < 0.05$) between the 100 µM Ni-treated plants versus the control plants for the non-hyperaccumulator species *A. thaliana, B. juncea* and metal-tolerant *S. farnsworthianus* as well as for the hyperaccumulator species *T. montanum* var. *siskiyouense*: ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$); respectively (Figure 10). There was no significant difference measured between the 100 µM Ni-treated plants

and control plants for the hyperaccumulator species *T. montanum* var. *montanum* and *S. polygaloides*: ($p = 0.37$, $p = 0.56$); respectively (Figure 10). There was a 0.08 fold decrease in *A. thaliana,* a 0.33 fold decrease in *B. juncea*, a 0.63 fold decrease in *S. farnsworthianus* as well as a 0.22 fold decrease in *T. montanum var. siskiyouense* 100 µM Ni-treated, Cu/ZnSOD bands as compared to the control bands (Figure 4).

Iron-Dependent Superoxide Dismutase Expression

Twelve days of Ni exposure resulted in a significant difference ($P<0.05$) between the 100 µM Ni-treated plants versus control plants for the non-hyperaccumulator species *A. thaliana*, *B. juncea*, and metal-tolerant *S. farnsworthianus* as well as for the hyperaccumulator species *T. montanum* var. *montanum*, *T. montanum* var. *siskiyouense*, and *S. polygaloides*: ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p < 0.01$, $p < 0.0001$, $p < 0.001$); respectively (Figure 11). There was a 0.20 fold decrease in *A. thaliana*, a 0.70 fold decrease in *B. juncea*, and a 1.32 fold increase in *S. farnsworthianus* 100 µM Ni-treated, FeSOD bands as compared to the control bands (Figure 5). There was also a 0.38 fold decrease in *S. polygaloides*, a 4.11 fold increase in *T. montanum* var. *montanum*, and a 0.24 decrease in *T. montanum* var. *siskiyouense* 100 µM Ni-treated, FeSOD bands as compared to the control bands (Figure 5).

Manganese-Dependent Superoxide Dismutase Expression

Twelve days of Ni exposure resulted in a significant difference ($P < 0.05$) between the 100 µM Ni-treated plants versus the control plants for the non-hyperaccumulator species *A. thaliana* and *B. juncea* and the hyperaccumulator species *T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense*: ($p < 0.01$, $p <0.0001$, $p < 0.05$, $p < 0.001$); respectively (Figure 12).

There was no significant difference measured between the 100 µM Ni-treated plants versus the control plants for the metal-tolerant species *S. farnsworthianus* and hyperaccumulator species *S. polygaloides*: ($p = 0.21$, $p = 0.76$; respectively (Figure 12). There was a 0.59 fold decrease in *A. thaliana*, a 1.61 fold increase in *B. juncea* as well as a 1.58 increase in *T. montanum* var. *montanum*, and a 0.33 fold decrease in *T. montanum* var. *siskiyouense* 100 µM Ni-treated, MnSOD bands as compared to the control bands (Figure 6).

ANOVAs

All control and 100 µM Ni-treated OSR protein (CAT, GR, MTP1, Cu/ZnSOD, FeSOD, and MnSOD) expression values for the non-accumulator species *A. thaliana*, *B. juncea*, and metal-tolerant *S. farnsworthianus* were pooled together as well as all the control and 100 µM Ni-treated OSR expression values for the hyperaccumulator species *T. montanum* var. *montanum*, *T. montanum* var. *siskiyouense*, and *S. polygaloides*.

The two-way ANOVA was conducted in order to determine if there was a significant effect of accumulator type (non-accumulator vs hyperaccumulator) and treatment on OSR expression and to determine if there was a significant interaction between the two. There was a significant effect ($P < 0.05$) of both accumulator type [$F(1, 284) = 7.24$, $p = 0.0076$] and treatment [$F(1, 284) = 19.95$, $p = 0.0001$] on total OSR expression. This indicates that there is a significant difference between non-accumulators and hyperaccumulators on OSR expression and that the Ni-treatment significantly affected total OSR expression. The two-way ANOVA also determined that there was a significant interaction ($P < 0.05$) between the effects of accumulator type and treatment [$F(1, 1) = 7.24$, $p = 0.0076$] on total OSR expression. The post-hoc Tukey HSD test determined that the 100 µM Ni-treated nonaccumulators were significantly different (p

< 0.05) to the control nonaccumulators, control hyperaccumulators, and the 100 µM Ni-treated hyperaccumulators.

The second two-way ANOVA was conducted to determine if there was a significant effect ($P < 0.05$) on OSR expression by OSR proteins and to determine if there was a significant interaction ($P < 0.05$) between OSR proteins and accumulator type. There was a significant effect by OSR proteins on OSR expression [$F (5, 284) = 3.27$, $p = 0.0069$] as well as significant interaction between accumulator type and OSR proteins [$F (1, 5) = 2.12$, $p = 0.062$]. The post hoc Tukey HSD test determined that there was a significant difference ($P <0.05$) between the hyperaccumulator FeSOD expression and the non-accumulator CAT, GR, MTP1 Cu/ZnSOD, MnSOD OSR and hyperaccumulator CAT, GR, MTP1 Cu/ZnSOD, FeSOD, MnSOD expressions.

The one-way ANOVA was conducted to determine if there was a significant difference in ($P < 0.05$) total SOD expression between species. There was a significant effect on SOD expression [$F (5,140) = 9.82$, $p = 0.0001$] and the post hoc test Tukey HSD test determined that there was a significant difference ($P < 0.05$) in total SOD expression in the hyperaccumulator species *T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense* as compared to all of the other plant species (Figure 13).

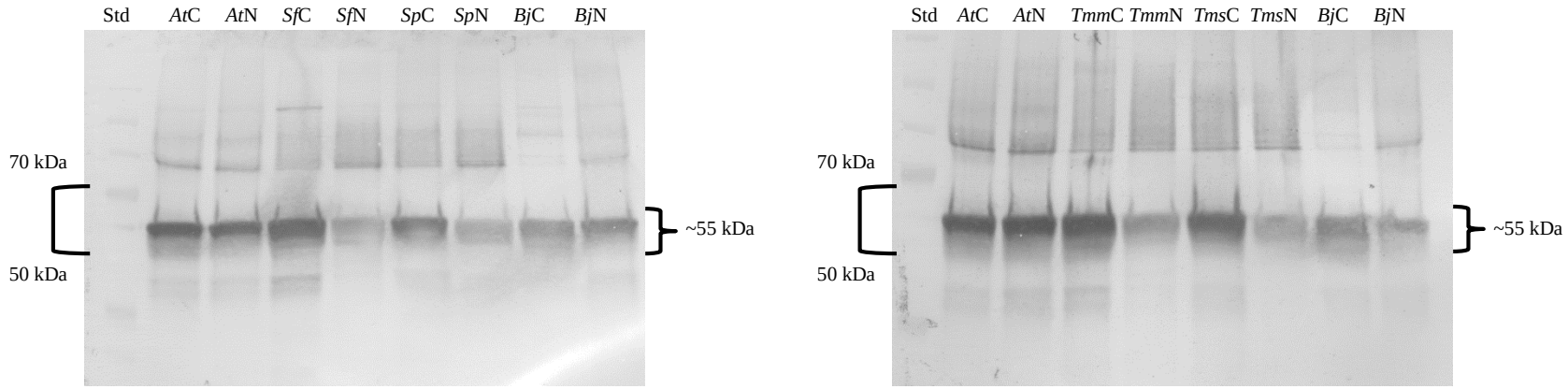

Figure 1: Nonquantitative expression of CAT. CAT (55 kDa) western blots from nonaccumulator and hyperaccumulator plant species using polyclonal CAT-specific antibodies designed from a KLH-conjugated peptide from *Arabidopsis thaliana* CAT-1, CAT-2, and CAT-3 isoforms.

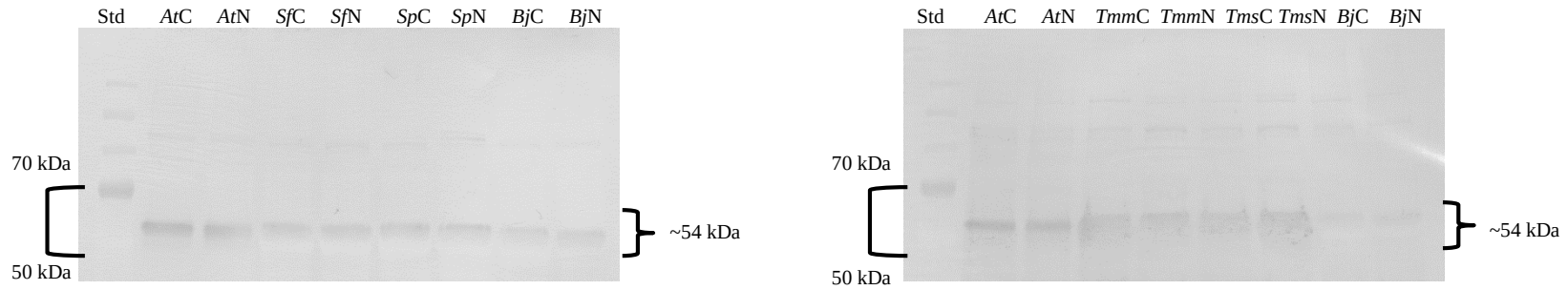

Figure 2: Nonquantitative expression of GR. GR (54 kDa) western blots from nonaccumulator and hyperaccumulator and hyperaccumulator plant species using polyclonal *Zea mays* GR-specific antibodies.

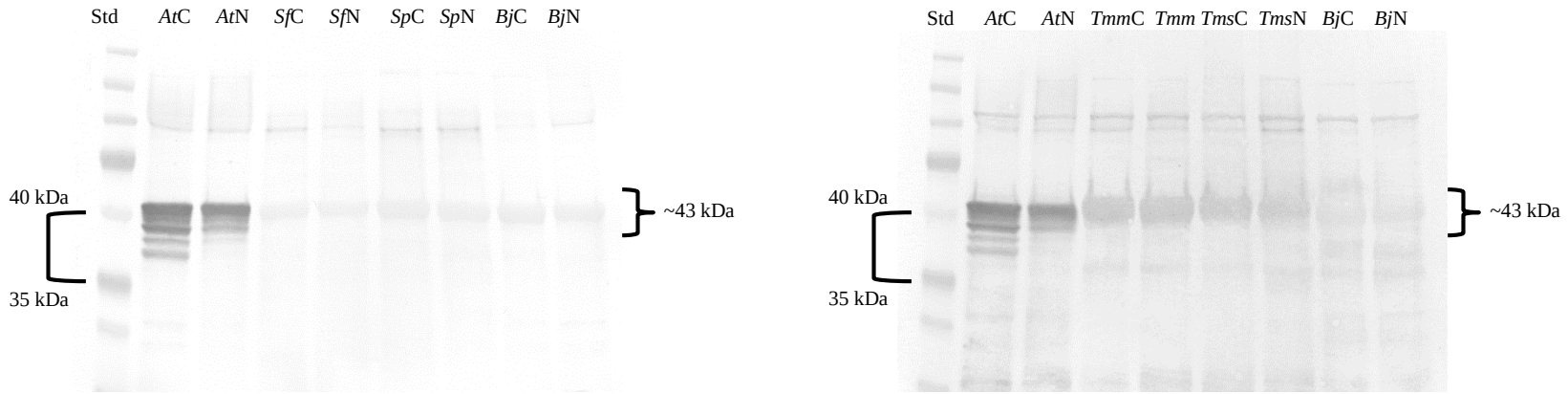

Figure 3: Nonquantitative expression of MTP1. MTP1 (43 kDa) western blots from nonaccumulator and hyperaccumulator plant species using polyclonal MTP1-specific antibodies designed from a KLH-conjugated synthetic peptide from *Arabidospsis thaliana*.

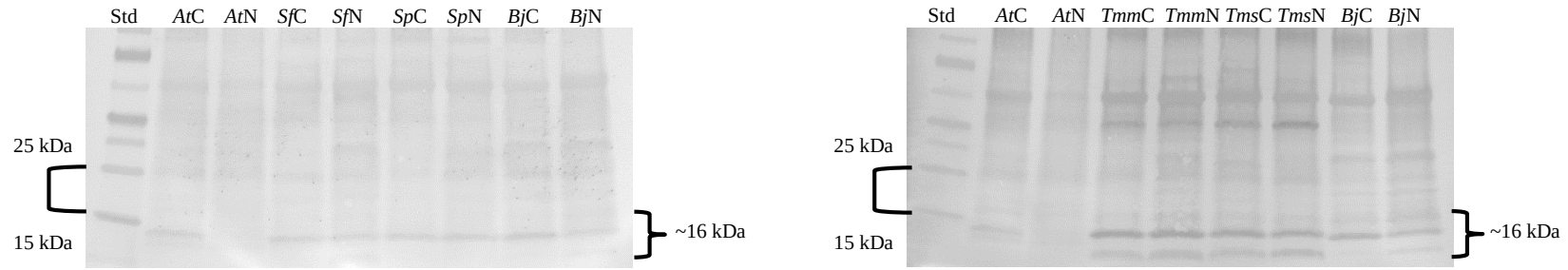

Figure 4: Nonquantitative expression of Cu/ZnSOD. Cu/ZnSOD (16 kDa) western blots from nonaccumulator and hyperaccumulator plant species using polyclonal cytosolic Cu/ZnSOD specific antibodies designed from a 15 amino acid synthetic peptide from *Olea europaea* (Olive).

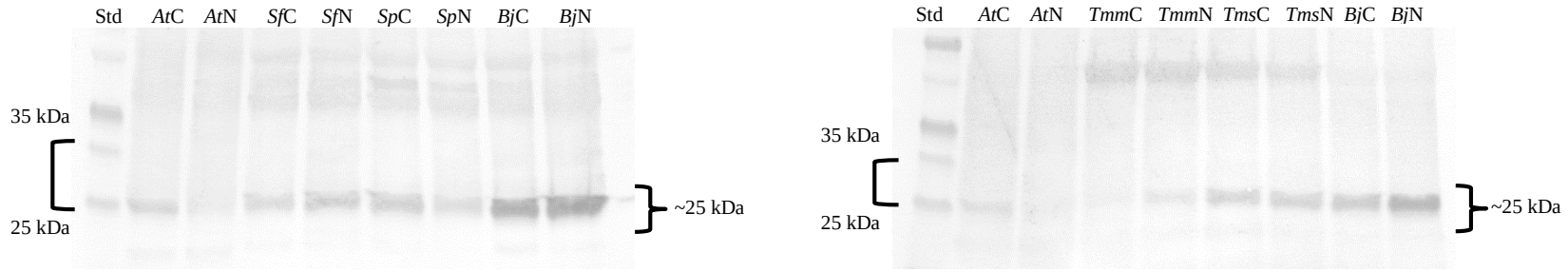

Figure 5: Nonquantitative expression of FeSOD. FeSOD (25 kDa) western blots from nonaccumulator and hyperaccumulator plant species using polyclonal *Chlamydomonas reinhardtii*-specific antibodies.

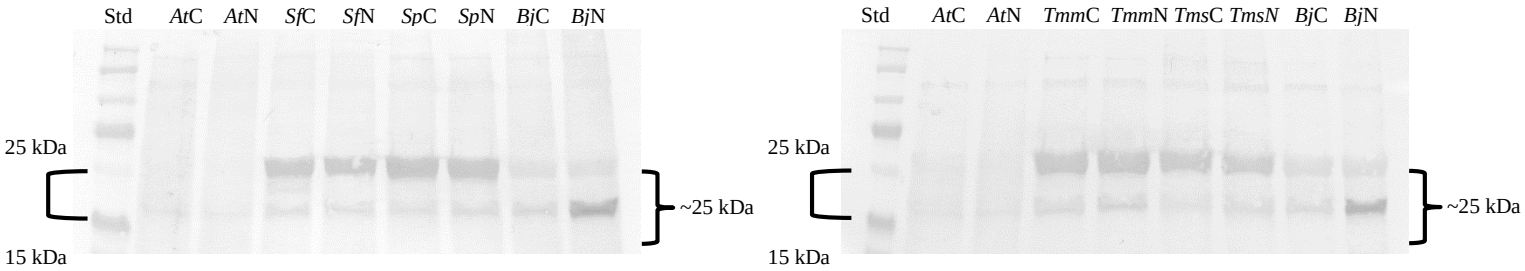

Figure 6: Nonquantitative expression of MnSOD. MnSOD (25 kDa) western blots from nonaccumulator and hyperaccumulator plant species using MnSOD-specific antibodies designed from a KLH-conjugated synthetic peptide from MnSOD sequences from di- and monocotyl plants.

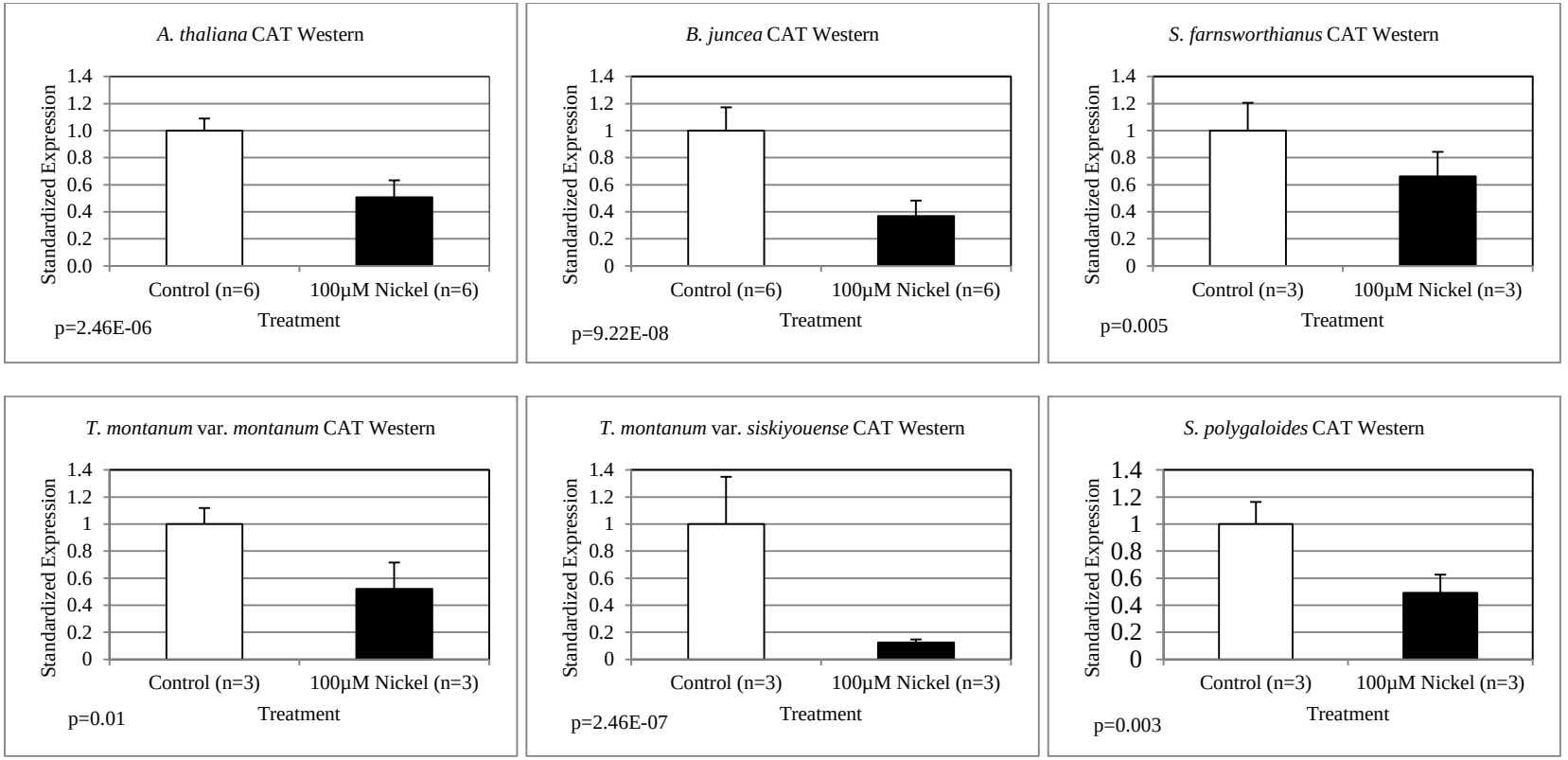

Figure 7: CAT expression of individual *Brassicaceae* species. Expression levels are standardized against their control. Top tier graphs illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

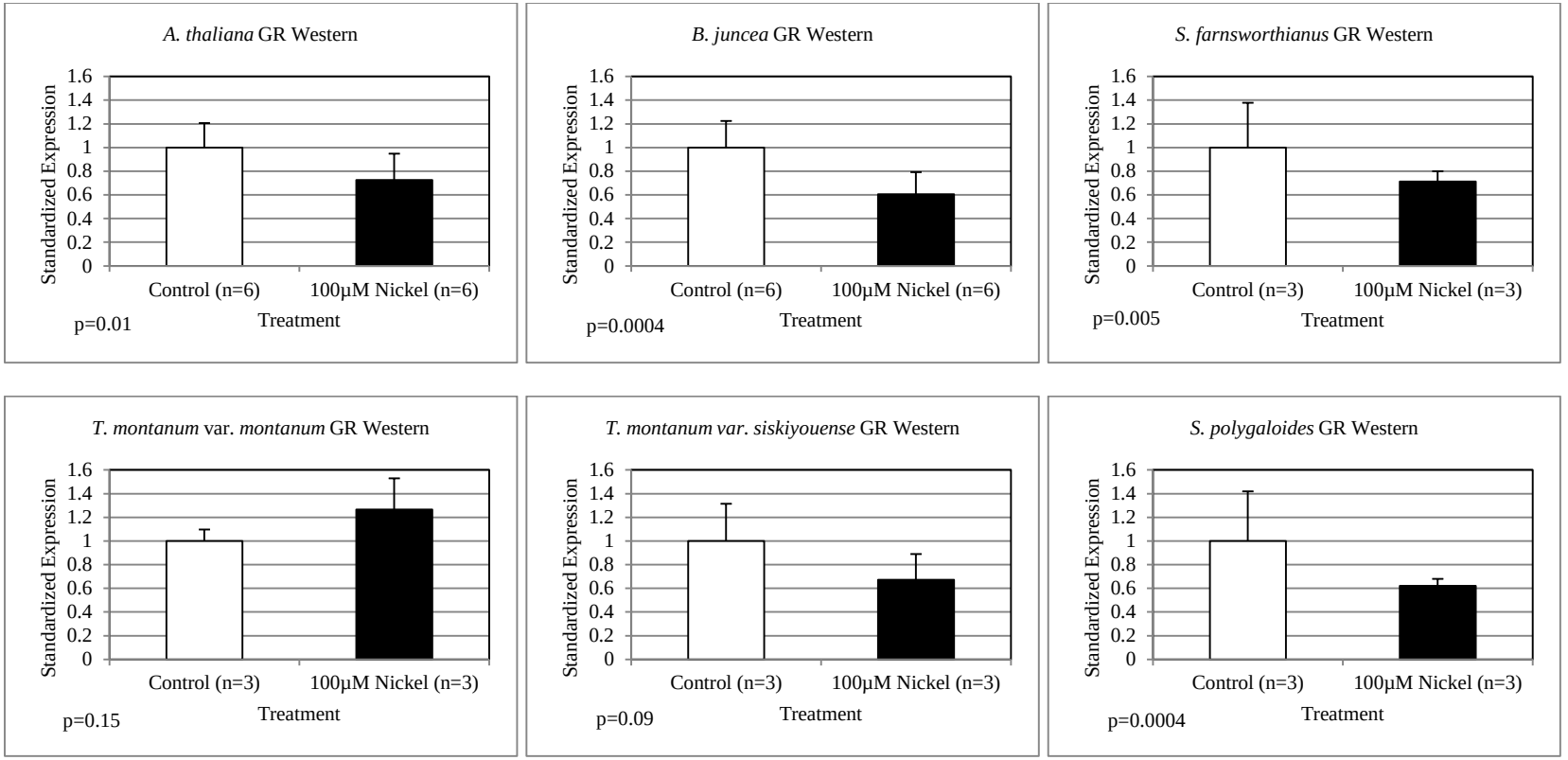

Figure 8: GR expression of individual *Brassicaceae* species. Expression levels are standardized against their control. Top tier graphs illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

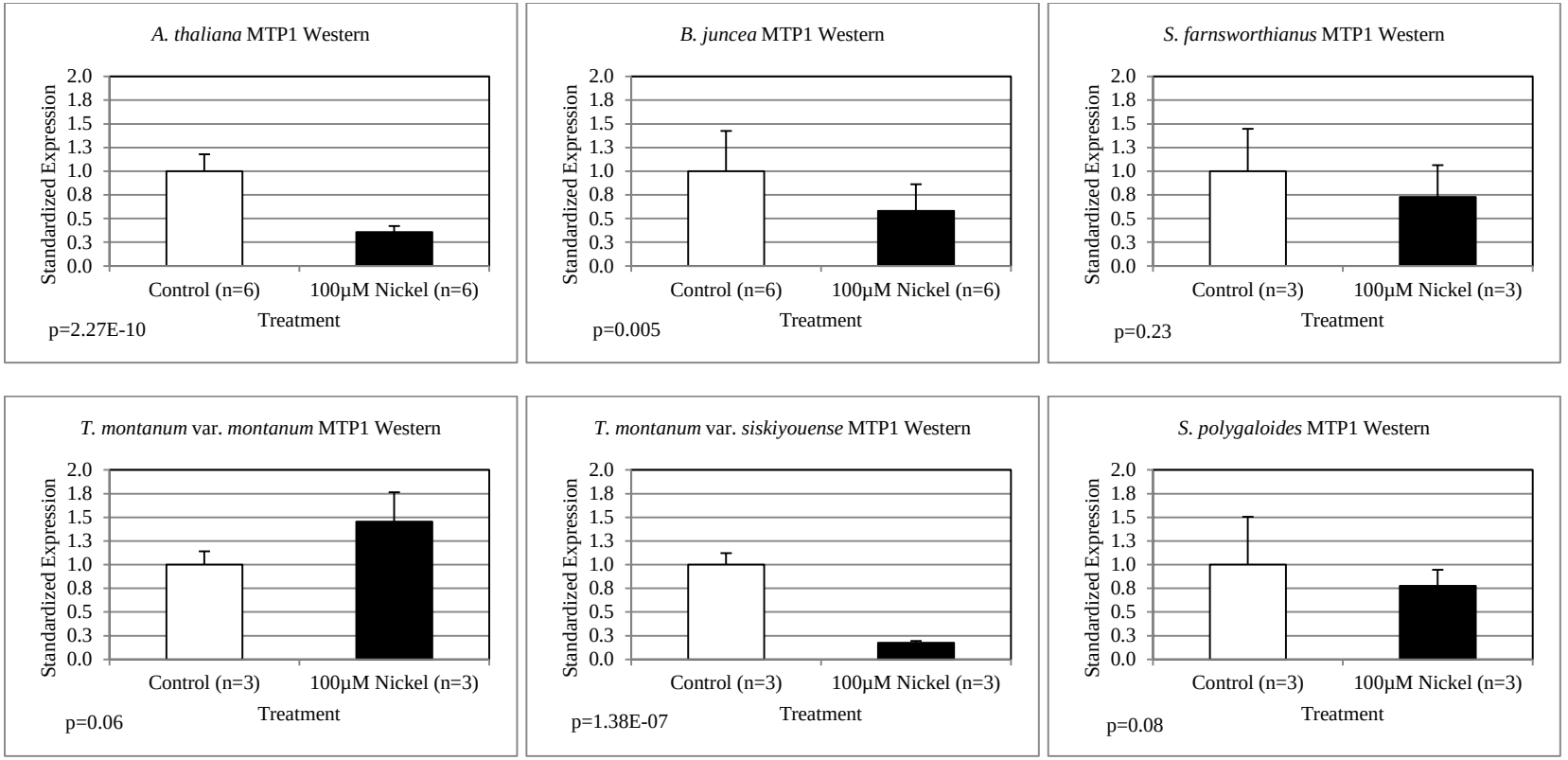

Figure 9: MTP1 expression of individual *Brassicaceae* species. Expression levels are standardized against their control. Top tier graphs illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

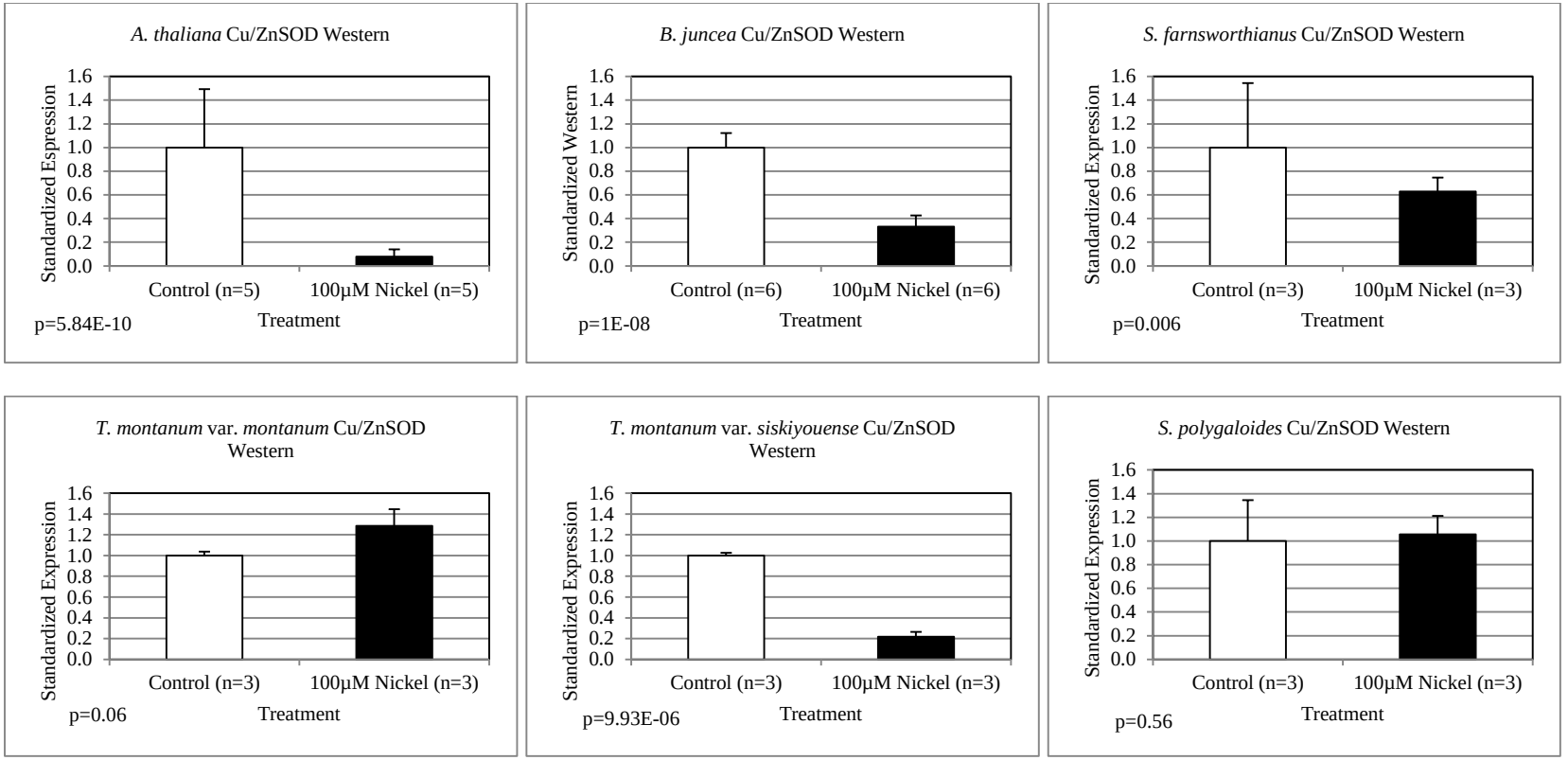

Figure 10: Cu/ZnSOD expression of individual *Brassicaceae* species. Expression levels are standardized against their control. Top tier graphs illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

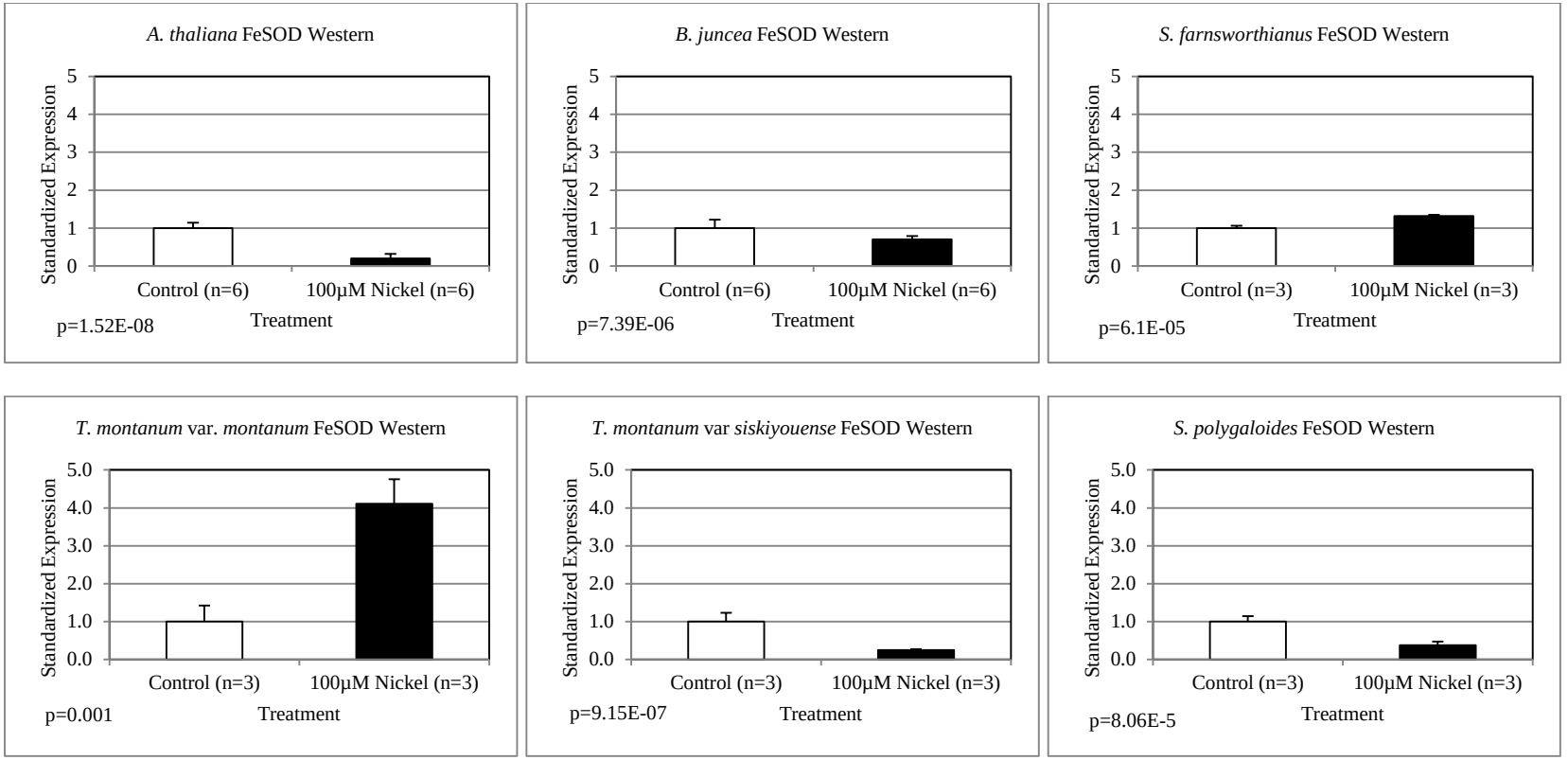

Figure 11: FeSOD expression of individual *Brassicaceae* species. Expression levels are standardized against their control. Top tier graph illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

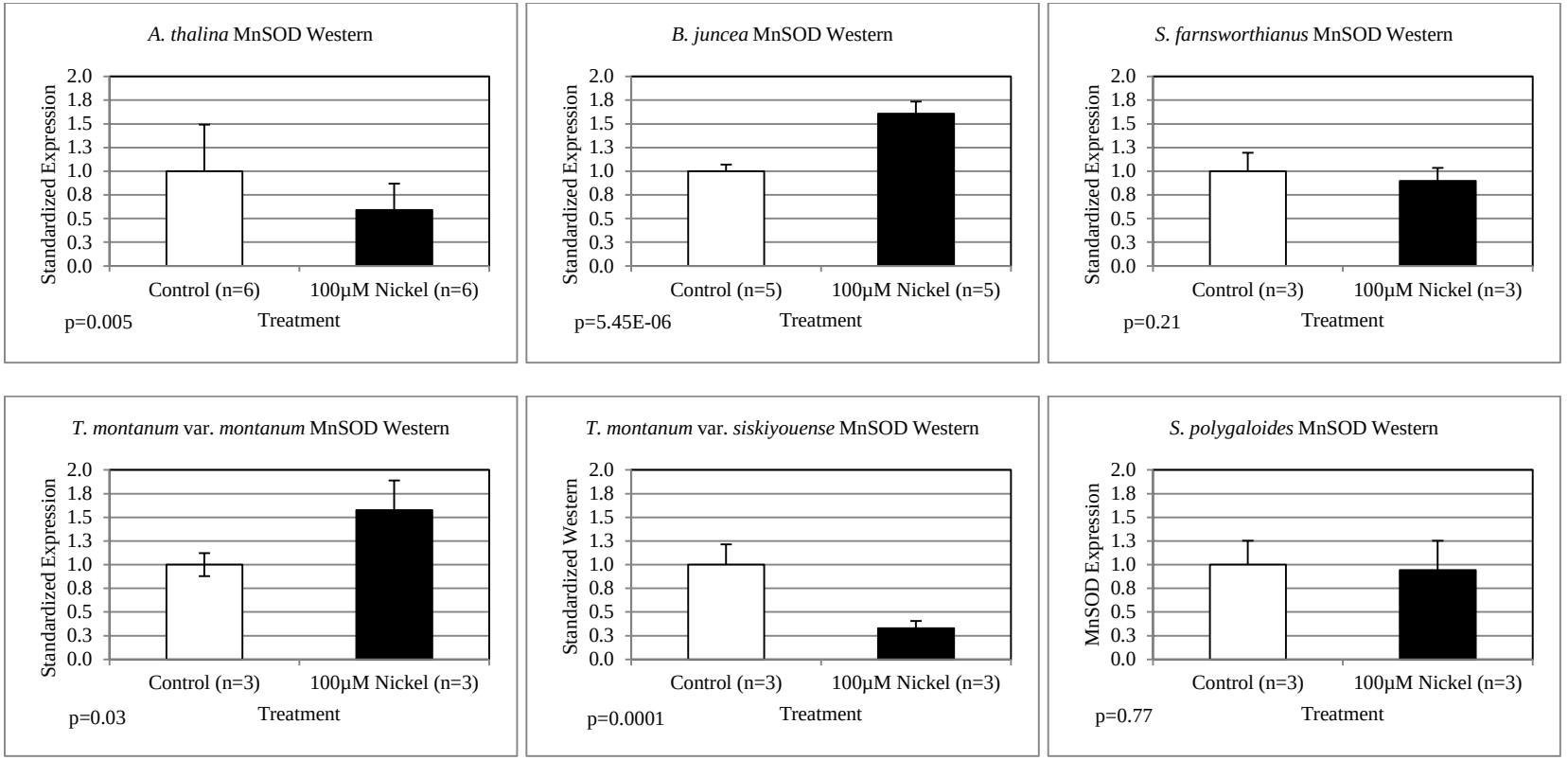

Figure 12: MnSOD expression of individual *Brassicaceae* species. Expression levels are standardized against their control. Top tier graphs illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

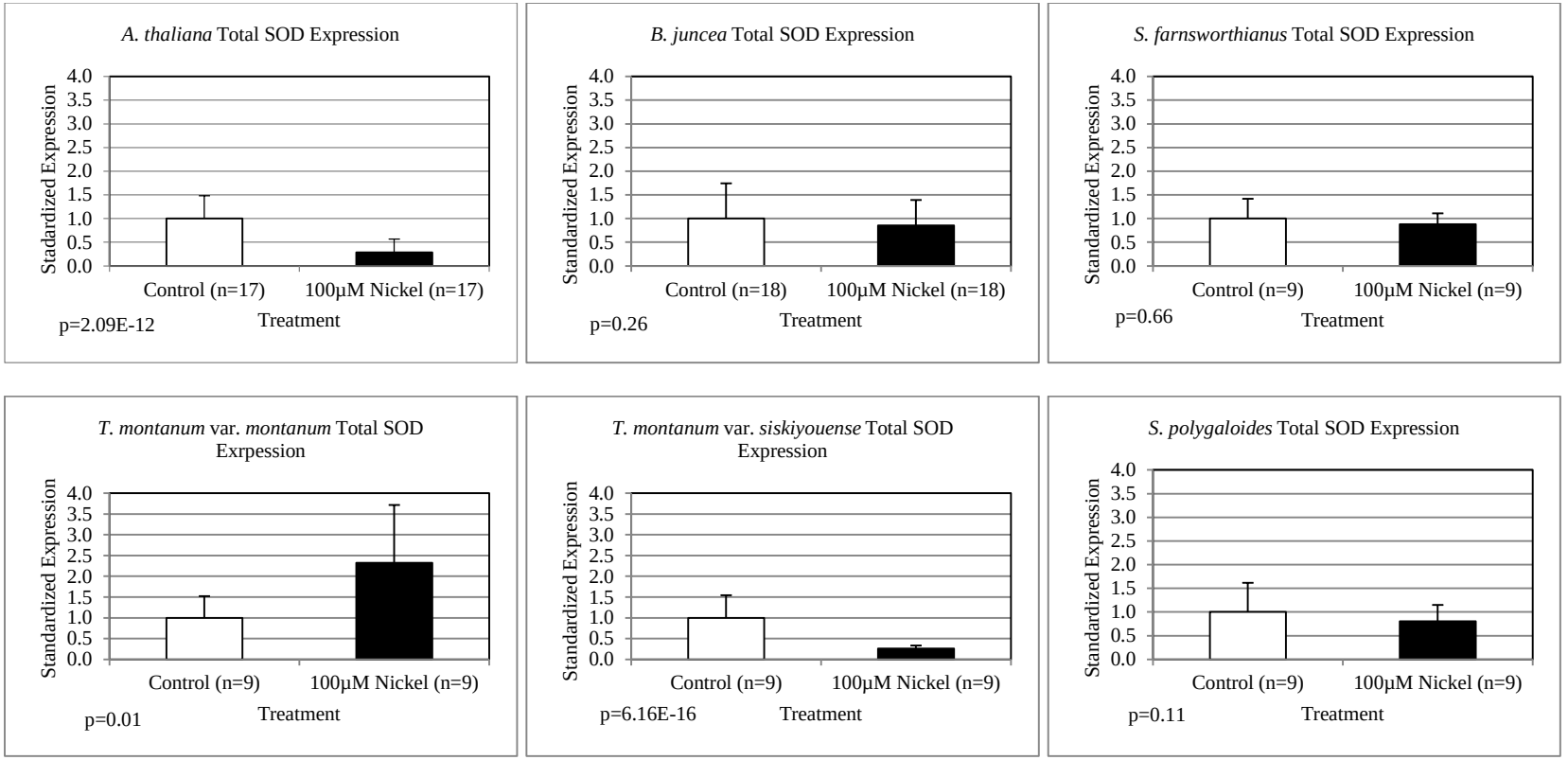

Figure 13: Combined SOD expressions of individual *Brassicaceae* species. All SOD (Cu/ZnSOD, FeSOD, and MnSOD) expressions are combined and expression levels are standardized against their control. Top tier graphs illustrate the nonaccumulator species or metal-tolerant and the bottom tier graphs illustrate the hyperaccumulator species.

CHAPTER IV

DISCUSSION

OSR per Species

Results from western blotting revealed that catalase protein expression was significantly down regulated in all of the nonaccumulator and hyperaccumulator plant species after being 100 µM Ni-treated for 12 days as compared to the controls. The hyperaccumulator species *T. montanum* var. *siskiyouense* showed the greatest expression deficit of all the plant species when 100 µM Ni-treated expressing on average only 12% ± 0.02 of that of the control. Interestingly, the related hyperaccumulator *T. montanum* var. *montanum,* although displaying a significant decrease, displayed the least down regulation of catalase when 100 µM Ni-treated expressing an average of 52% ± 0.19 of that of the control. The significant down regulation of catalase in all of the plant species may suggest that this particular OSR enzyme is sensitive to metal exposure or that its role in the decomposition of hydrogen peroxide may be effectively substituted by other molecules with similar functions such as glutathione peroxidase (GPX) (Navrot et al. 2006) and ascorbate peroxidase (APX) (Caverzan et al. 2012).

Glutathione reductase was significantly down regulated in the nonaccumulator species *A. thaliana, B. juncea,* and metal-tolerant *S. farnsworthianus*, as well as the hyperaccumulator species *S. polygaloides* when 100 µM Ni-treated for 12 days as compared to the controls. This may indicate that their GRs are sensitive to metal toxicity or that they may not play a significant role in the hyperaccumulator species for conferring resistance to metal toxicity. There was no

statistically significant difference in expression in the hyperaccumulator species *T. montanum* var. *montanum* and *T. montanum* var. *siskiyouense* after treatment as compared to the controls indicating that their GR may not be as sensitive to metal toxicity or that some other factors may be involved in sustaining their active GR pools.

MTP1 was significantly down regulated in the 100 µM Ni-treated nonaccumulator species *A. thaliana, B. juncea* and in the hyperaccumulator species *T. montanum* var. *siskiyouense* as compared to their controls. It is interesting to note that the hyperaccumulator species *T. montanum* var. *siskiyouense* displayed the lowest MTP1 expression at 18% ± 0.02 of that of the control when 100 µM Ni-treated followed by the nonaccumulator *A. thaliana* at 36% ± 0.06 when 100 µM Ni-treated as compared to the control. There was no statistically significant difference in expression in the 100 µM Ni-treated metal-tolerant species *S. farnsworthianus* as well as the hyperaccumulator species *T. montanum* var. *montanum* and *S. polygaloides* as compared to their controls. This may indicate that the baseline expression of MTP1 in these species may be adequate to handle metal-induced oxidative stress when 100 µM Ni-treated and up-regulation of the gene may not have been necessary. The polyclonal anti-AtMTP1 antibody used for MTP1 detection was not designed to specifically detect other MTP variants such as MTPA1, MTPA2, MTP3, and MTPB (Agrisera, Vännäs, SE). It has been previously demonstrated that over expression of MTP3 increases Zn accumulation and tolerance in *A. thaliana* (Arrivault et al. 2006) and it may be possible that these related metal tolerance proteins may have been induced by 100 µM Ni-treatment in response to oxidative stress.

The copper-zinc dependent SOD was significantly down regulated in all of the 100 µM Ni-treated nonaccumulator species as compared to their controls as well as in the hyperaccumulator species *T. montanum* var. *siskiyouense*. *A. thaliana* exhibited the most down-

regulation of Cu/ZnSOD with the 100 µM Ni-treated set averaging 0.08% ± 0.06 expression of that of the control set. This may be due to the lack of metal tolerance that the nonaccumulator plant species exhibit and their down regulation may be indicative of deteriorating health due to Ni-induced oxidative stress. The hyperaccumulator species *T. montanum* var. *siskiyouense* was significantly reduced with the 100 µM Ni-treated set averaging 22% ± 0.05 expression of that of the control set. There was no significant difference in Cu/ZnSOD expression in the 100 µM Ni-treated hyperaccumulator species *T. montanum* var. *montanum* and *S. polygaloides* as compared to their controls with *S. polygaloides* demonstrating the least change with a 6% ± 0.16 increase in expression in the 100 µM Ni-treated set as compared to the control set. It is interesting to note that the nonaccumulator species *A. thaliana, B. juncea,* and the hyperaccumulator species *T. montanum* var. *siskiyouense* displayed similar trends in Cu/ZnSOD and MTP1 regulation when 100 µM Ni-treated as compared to their respective controls; the 100 µM Ni-treated sets were all significantly down regulated as compared to their respective controls. This may indicate that Cu/ZnSOD and MTP1 regulation may be interdependent or that they may share a response mechanism when 100 µM Ni-treated. MTP1 is a member of the cation diffusion facilitator family responsible for transporting Zinc into leaf vacuoles (Kobae et al. 2004) and it has been previously demonstrated by Kobayashi *et al.* (1996) that the related ZRC1 gene is responsible for conferring oxidative stress tolerance. It may be probable that an interaction may exist between the translocation of Zn by MTP1 and its use as a metallic cofactor in Cu/ZnSOD that may be responsible for their similar expressions within these species in response to oxidative stress.

The expression of the iron-dependent SOD was significantly influenced by 100 µM Ni-treatment in all of the nonaccumulator and hyperaccumulator species as compared to their controls. The nonaccumulator species *A. thaliana* and *B. juncea*, and hyperaccumulator species

T. montanum var. *siskiyouense* and *S. polygaloides* displayed significant down regulation of FeSOD when 100 µM Ni-treated as compared to the controls. *A. thaliana* exhibited to most down regulation of FeSOD expression with the 100 µM Ni-treated set averaging 20% ± 0.12 expression of that of the control set followed by *T. montanum* var. *siskiyouense* at 24% ± 0.03 expression of that of the control set. The 100 µM Ni-treated metal-tolerant species *S. farnsworthianus* and hyperaccumulator species *T. montanum* var. *montanum* both exhibited significant up regulation of FeSOD as compared to their controls. Interestingly, T. *montanum* var. *montanum* displayed the highest up-regulation when 100 µM Ni-treated with 411% ± 0.65 increase in expression of FeSOD as compared to the controls. This was the largest increase in expression out of all of the OSR proteins from any 100 µM Ni-treated species. Conversely, the related hyperaccumulator *T. montanum* var. *siskiyouense* displayed a significant down regulation as previously shown in Figure 11.

The expression of the manganese-dependent SOD was significantly down-regulated in the nonaccumulator species *A. thaliana* and hyperaccumulator species *T. montanum* var. *siskiyouense* with the 100 µM Ni-treated sets averaging 59% ± 0.27 and 33% ± 0.08 expression of that of their controls, respectively. Conversely, MnSOD expression was significantly up-regulated in the nonaccumulator species *B. juncea* and hyperaccumulator species *T. montanum var. montanum* averaging a 61% ± 0.13 and 68% ± 0.32 increase in expression in the 100 µM Ni-treated sets as compared to their controls, respectively. The metal-tolerant species *S. farnsworthianus* and hyperaccumulator species *S. polygaloides* displayed no significant change in MnSOD regulation in the 100 µM Ni-treated sets as compared to their controls.

The 100 µM Ni-treated *A. thaliana* exhibited significant down regulation of all OSR proteins as compared to the controls as well as *T. montanum* var. *siskiyouense* with the exception

of GR which exhibited no significant change in expression as compared to the control. The 100 µM Ni-treated nonaccumulator species *B. juncea* and hyperaccumulator species *T. montanum* var. *montanum* had an increase in FeSOD expression as compared to their controls.

Total SOD Expression

The post hoc Tukey HSD test determined that there was a significant difference in regulation of SODs when 100 µM Ni-treated in the hyperaccumulator *T. montanum* var. *montanum* ($P < 0.05$) when compared to all of the other species. *T. montanum* var. *montanum* displayed a significant increase in total SOD expression. Interestingly, *T. montanum* var. *siskiyouense* displayed to opposite results. The 100 µM Ni-treated *T. montanum* var. *siskiyouense* significantly ($P < 0.05$) down regulated SOD expression when compared to all of the other species. The nonaccumulator species *A. thaliana*, *B. juncea*, and *S. farnsworthianus*, and the hyperaccumulator species *S. polygaloides* displayed no significant difference in SOD regulation as compared to one another.

Nonaccumulator vs. Hyperaccumulator Total OSR Expression

The two-way ANOVA determined that there was a significant interaction ($P < 0.05$) between treatment and accumulator type. The post hoc Tukey HSD determined that the 100 µM Ni-treated, nonaccumulator group displayed significant down regulation ($P < 0.05$) of all the OSR proteins as compared to the control, nonaccumulator; 100 µM Ni-treated, hyperaccumulator; and control, hyperaccumulator groups. The rest of the groups showed no significant difference in total OSR protein expression from one another as indicated by the post hoc Tukey HSD test. These results may indicate that hyperaccumulators may not be over-

expressing OSR proteins as a mechanism to prevent or alleviate metal-induced oxidative stress, but instead may have the phenotypic ability to maintain a steady OSR protein expression under increased oxidative assault.

Conclusion

The results of the western blots indicate that expression of OSR proteins are significantly affected in both the nonaccumulator, metal-tolerant and hyperaccumulator species in response to 100 µM Ni-treatment. The CAT western blots revealed significant down regulation of this enzyme in all 100 µM Ni-treated species and as such may be an appropriate bio-indicator of oxidative stress. The role of CAT in scavenging H_2O_2 may be substituted by APX, GPX, and various peroxiredoxins during CAT depleted circumstances such as those induced by increased ROS production. Characterization of APX and GPX through western blots may reveal a relationship between the scavenging of H_2O_2 by APX and GPX during oxidative stress. CAT is predominantly scavenges photorespiratory H_2O_2 while APX is localized within chloroplasts (Willekens et al. 1997) and further studies on the distribution of CAT within cells may reveal where the effects of ROS are most prominent. Genome sequencing of *A. thaliana* has also revealed that there are three *CAT* genes with high similarity between all three polypeptides (Frugoli et al. 1996). Although there are variations in the localization of CAT isoforms between species, CAT-1, CAT-2, and CAT-3 isoforms are generally associated with photosynthetic, vascular, and reproductive tissues, respectively (Willekens et al. 1995). The polyclonal anti-CAT antibody used in this study was designed from KLH-conjugated peptides consisting of AtCAT-1, AtCAT-2, and AtCAT-3 and did not differentiate between these isoforms. Characterization of

the individual isoforms may help better distinguish the effects of metal-induced oxidative stress on CAT regulation.

GR expression was significantly down regulated when 100 µM Ni-treated in the nonaccumulator species as well as in *S. polygaloides* with no significant change in expression within *T. montanum* var. *montanum* and var. *siskiyouense* as compared to their respective controls. Similar to CAT, these results suggest that GR pools may be a bio-indicator of metal-induced, oxidative stress. GR has been shown to exist as multiple isoforms that are compartment-specific with a majority of GR being localized in chloroplasts (Edwards et al. 1990). The polyclonal anti-GR IgG antibody used for GR detection recognized both chloroplastic and cytoplasmic forms and did not differentiate between the varying isoforms due to their variability only occurring in their isoelectric points and not molecular weight as determined by Edwards *et al.* (1990). Glutathione and related enzymes have been demonstrated to increase as a stress response in publications compiled and reviewed by Tausz *et al.* (2004) and the lack of differentiation in GR isoforms may account for the results in this study. Independent characterization of the two localized forms of GR can help better evaluate the response mechanism of metal accumulation between the nonaccumulator and hyperaccumulator plant species.

MTP1 expression was significantly down-regulated in the 100 µM Ni-treated nonaccumulator species *A. thaliana* and *B. juncea* as well as in the hyperaccumulator species *T. montanum* var. *montanum*. Surprisingly, there was no significant change in MTP1 regulation in the hyperaccumulator species *T. montanum* var. *montanum* and *S. polygaloides* as well as in the metal-tolerant species *S. farnsworthianus*. The variation in MTP1 expression between and within the nonaccumulator and hyperaccumulator groups may be due to the induction of related proteins

such as MTPA1, MTPA2, MTP3, and MTPB in response to metal-induced, oxidative stress. Induction of MTP-related ZRC1 by the MAPK-activated protein kinase RCK2 in response to oxidative stress has been previously demonstrated by Bilsland *et al*. (2004) in *S. cerevisiae* and suggests that oxidative stress induces expression of genes responsible for Zn translocation. Characterization of the varying MTP genes involved in Zn transport may aid in identifying a possible role that MTPs perform in response to metal-induced, oxidative stress.

The western blots for Cu/Zn, FeSOD, and MnSOD revealed that SOD expression in *A. thaliana* and *T. montanum* var. *siskiyouense* were significantly decreased in the 100 µM Ni-treated groups when compared to their respective controls. The nonaccumulator species *A. thaliana* demonstrated sensitivity to each 100 µM Ni-treatment. These results support its use as a model organism in heavy metal tolerance by serving as a basis for comparison against hyperaccumulator species within the *Brassicaceae* family. Interestingly, the metal-tolerant species *S. farnsworthianus* displayed a significant decrease in Cu/ZnSOD regulation and a significant increase in FeSOD regulation with Cu/ZnSOD being expressed at a level of 63% ± 0.12 of that of the control group and FeSOD being expressed at a level of 132% ± 0.14 of that of the control group. *T. montanum* var. *montanum* displayed the only significant up-regulation of total SOD when 100 µM Ni-treated as compared to its control. Specifically, FeSOD displayed the most significant up regulation at a level of 411% ± 0.65 of that of the control group followed by MnSOD being expressed at a level of 158% ± 0.32 of that of the control group. There was no significant difference in Cu/ZnSOD expression by *T. montanum* var. *montanum* but it is interesting to note that there was a slight increase in expression in the 100 µM Ni-treated group as compared to the control group.

Similarly to the H_2O_2 scavenging enzymes APX, CAT, and GPX, the varying SODs are generally compartmentalized within the cell. FeSOD is localized within the chloroplast, MnSODs are found in the mitochondria and peroxisomes, while Cu/ZnSODs are found within the chloroplast and the cytosol (Alscher et al. 2002). The subcellular localization of the varying SODs may explain the differences in their responses, and similarly to CAT, may reveal where the effects of ROS are most apparent. In this case, the results seem to suggest that oxidative stress may originate within chloroplasts due to the significant variation in chloroplastic FeSOD response in all of the nonaccumulator and hyperaccumulator species when 100 µM Ni-treated as compared to their respective controls. Chloroplasts house the photosystem I and photosystem II centers which generate large amounts of ROS through the Mehler's reaction (Asada 2006) so it may be concluded that FeSOD may have a more significant role in oxidative stress response.

It has been previously demonstrated by Into (2010) that the rate of enzymatic activity of CAT, GR, and SOD varies in nonaccumulator and hyperaccumulator plant species. Into's research shows that GR and SOD enzymatic activity within the nonaccumulator species *A. thaliana* and *B. juncea* as well as in the hyperaccumulator *T. montanum* var. *montanum* is significantly increased in the 100 µM Ni-treated groups as compared to their respective controls. These results are in contrast to the significant decrease in GR and SOD expression in the nonaccumulator species *A. thaliana* and *B. juncea*. Interestingly, these results suggest that the GR and SOD enzymes from *A. thaliana*, *B. juncea*, and *T. montanum* var. *montanum* are not being inhibited by 100 µM Ni-treatment but significant down regulation of their protein expression is still occurring. The hyperaccumulator *T. montanum* var. *montanum* has been found to increase both SOD enzymatic activity as shown by Into (2010) as well as SOD protein expression. With the exception of *T. montanum* var. *montanum*'s SOD enzymatic activity, Into's

research also demonstrates that there is no significant change in the rate of CAT, GR, and SOD enzymatic activity in the hyperaccumulator species *T. montanum* var. *montanum, T. montanum* var. *siskiyouense*, and *S. polygaloides* after 100 µM Ni-treatment as compared to their respective controls. It is interesting to note that although there is no significant difference in enzymatic activity of CAT within the hyperaccumulator species, the baseline enzymatic activity was 2.5 to 8 folds greater than that of the nonaccumulator species. Similar to the sustained OSR expression within hyperaccumulator plants after 100 µM Ni-treatment, the sustained baseline enzymatic activity of OSR proteins within the hyperaccumulators may be a possible mechanism employed by hyperaccumulators to resist metal-induced oxidative stress.

The ability of a plant to hyperaccumulate heavy metals and manage oxidative stress is achieved through a consortium of mechanisms. Modulation of OSR protein expression is one of many mechanisms that allows hyperaccumulators to manage oxidative stress when exposed to metalliferous soils and multiple physiological mechanisms involved in the exclusion, translocation and sequestration of heavy metals also play a role in conferring metal tolerance. Further investigation of these mechanisms is necessary in order to engineer an ideal hyperaccumulator plant for the purposes of phytoremediation through phytoextraction.

The objective of this study was to provide characterization of key proteins involved in ROS management induced by 100 µM Ni-treatment in nonaccumulator and hyperaccumulator plant species. This study shows that there is a relationship between metal hyperaccumulators in certain *Brassicaceae* plant species and varying OSR protein expression as a mechanism responsible for this phenotype. Expanding the investigation to include related antioxidant response proteins and molecules such as ascorbate peroxidase, glutathione peroxidase, and

glutathione as well as distinguishing expression between varying isoforms and their cellular locations can provide a better depiction of OSR expression in conferring metal tolerance.

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BIOGRAPHICAL SKETCH

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