5-2012

Assessment of Oxidative Stress Gene Expression in Brassicaceae Plant Species Using A Real-Time PCR Method

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ASSESSMENT OF OXIDATIVE STRESS GENE EXPRESSION IN BRASSICACEAE PLANT SPECIES USING A REAL-TIME PCR METHOD

A Thesis

by

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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

May 2012

Major Subject: Biology

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May 2012

ABSTRACT

Ruiz, Florestella, Assessment of Oxidative Stress Gene Expression in Brassicaceae Plant Species Using a Real-Time PCR Method. Master of Science (MS), May, 2012, 33 pp., 9 tables, 24 references.

Many plant species in the *Brassicaceae* plant family possess the ability to accumulate heavy metals. One challenge in using these hyperaccumulator plants for phytoremediation is that they are slow growing and often have a low biomass. This is a limitation should they be utilized for phytoremediation. However, the mechanisms by which hyperaccumulators tolerate heavy metals are of interest as these characteristics can be engineered into a plant species that is more suited for use in phytoremediation. Therefore, modification of oxidative stress response (OSR) gene expression in *Brassicaceae* species has become of great interest. Oxidative stress gene induction in both non-accumulator and hyper-accumulator plant species can be detected by the Real-Time Polymerase Chain Reaction method. Fold differences in gene expression between non accumulator and hyperaccumulator plant species can be compared to enzymatic activity assays to determine a correlation between OSR gene expression levels and OSR enzyme activity.

DEDICATION

For my parents, Rogelio and Virginia Ruiz, and their unconditional love, support, and motivation. For my sisters, Liza, Virginia, and Alma, who remind me to laugh at my mistakes. For my dear husband Mark, and his unwavering patience, love, and understanding.

ACKNOWLEDGMENTS

I will always be grateful to Dr. Michael Persans, chair of my thesis committee, for all his mentoring and advice. From research design, data interpretation, to thesis editing, he encouraged me to complete this process through his infinite patience and guidance. My thanks go to my thesis committee members: Dr. Matthew Terry and Dr. Jonathan Lieman. Their advice, input, and comments on my thesis helped to ensure the quality of my intellectual work.

I would also like to thank my colleagues and coworkers at the UTPA biology research lab that helped me with the lab equipment. Special thanks to Tom Eubanks and Fritzie Into, who never hesitated to help me if I had a question over a certain procedure or equipment. Many thanks to Yessica Cerino and Elyssa Garza for their support when I needed the extra hands with an experiment.

I am also grateful for The University of Texas-Medical Branch Bridges to Ph.D program and USDA for their funding of my research.

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CHAPTER I

INTRODUCTION

While many plant species have been identified as having the ability to tolerate and accumulate heavy metals (hyperaccumulators), these plants are not practical for use in phytoremediation because they are often slow-growing and do not possess a large enough biomass to be exploited for use by this technology (Pilon-Smits & LeDuc, 2009). It is speculated that the expression of certain oxidative stress response (OSR) genes confer to hyperaccumulators their ability to tolerate and accumulate some heavy metals (Hong-Bo, *et al*, 2010). OSR genes of interest examined in this study, Superoxide Dismutase (Cu/Zn, Fe, and Mn), Catalase, and Glutathione Reductase, may contribute to plants ability to tolerate and accumulate heavy metals. These genes, also known as oxidative stress response genes, act by neutralizing reactive oxygen species either by detoxifying reactive oxygen species or producing compounds that function as antioxidant molecules.

The oxidative stress response genes that were analyzed in this study are Superoxide Dismutase (Cu/Zn, Mn, and Fe), Catalase, and Glutathione Reductase. Superoxide Dismutase functions by catalyzing the breakdown of superoxide to hydrogen peroxide and water (Park, 2005). Catalase functions by catalyzing the degradation of two molecules of hydrogen peroxide into oxygen and water (Chelikani, *et al*, 2003). Glutathione Reductase functions by converting oxidized glutathione (glutathione disulfide, or GSSH) to GSH, the reduced form of glutathione

thus recycling the molecule for further use in detoxifying additional oxygen radicals (Romero-Puertas, *et al*, 2006).

Statement of the problem

Heavy metal contamination in the environment is largely the result of human activity, such as mining, agriculture, and the creation and use of synthetic products containing heavy metals (Leyval, *et al*, 1997). The presence of heavy metals in the environment is detrimental to organisms and ecosystems because many organisms do not have the ability to eliminate heavy metals once ingested. Many heavy metals, such as Cd and Pb, can biomagnify up the food chain, which leads to contaminated food sources for humans and domesticated animals (Kamaruzzaman, 2012).

Current methods of remediating contaminated sites involve excavation, which can be done *in situ* or *ex situ*. *In situ* excavation is the treatment of soil on-site; while *ex situ* is the removal and treatment of the heavy metal waste off-site (Khan *et al*, 2000). Excavation, however, is not practical because it is expensive for very large areas, costing upwards of $1 million per acre, and it is risky, because the transportation of the contaminated material is prone to spreading from the site of origination to its final destination.

One method that is cheaper and more environmentally friendly is phytoremediation, which is the remediation of a contaminated area using vegetation (Khan *et al*, 2000). Some plant species are able to selectively uptake certain elements via their roots and shoots, and accumulate them in certain sub-cellular locations within the plant, such as the vacuole (Cheng 2003). Select plant species of the *Brassicaceae* family have the ability to tolerate and accumulate heavy metals

from their environment (Peer *et al*, 2003). However, many of these plants are slow-growing and lack enough biomass to be exploited on an industrial scale. The ideal plant for use with phytoremediation technology should be fast-growing while maintaining the ability to accumulate a significant amount of its biomass as heavy metals (Pulford *et al*, 2002).

Conceptual Framework

The goal of this thesis is to determine the difference in expression levels of the oxidative stress response genes Superoxide Dismutase, Catalase and Glutathione Reductase in *Arabidopsis thaliana* and *Thlaspi montanum var. montanum* under control conditions and in response to treatment with 100 µM Ni.

Twelve day control and 100 µM nickel acetate treated non-accumulator (*Arabidopsis thaliana*) and hyper-accumulator (*Thlaspi montanum var. montanum*) plant tissue samples were ground in liquid nitrogen. The total RNA of each species, control and treated, were then isolated. These total RNAs were then DNAse treated to remove any genomic DNA. First strand cDNA synthesis was performed on each species and treatment using gene-specific primers for our genes of interest; Superoxide Dismutase Cu/Zn (SOD Cu/Zn), Superoxide Dismutase Fe (SOD Fe), Superoxide Dismutase Mn (SOD Mn), Catalase (CAT), and Glutathione Reductase (GR) as well as the reference gene, Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH). These cDNAs were amplified via Real-Time Polymerase Chain Reaction analysis. The presence of the proper amplicons was determined by running a 1% agarose gel on traditional polymerase chain reaction products. Additionally, these traditional PCR products were cloned and sequenced to determine their true identities to confirm that what were being amplified by the Real-Time PCR analysis

were in fact the OSR genes in question. The relative expression levels of the mRNA from the various OSR genes were determined by calculating the fold difference between the levels of expression of each gene using Real-Time PCR data.

Research Questions and Hypothesis

The purpose of this study was to compare the expression levels of oxidative stress genes in *Arabidopsis thaliana* and *Thlaspi montanum var. montanum* under control conditions and treatment with 100 µM Ni for twelve days. The mRNA expression data obtained from Real-Time Polymerase Chain Reaction using *Brassicaecae* universal primers was also compared with protein enzymatic activities of these oxidative stress response genes as well as the post treatment nickel content of the plant leaves.

The hypothesis tested in this thesis is that in comparison to non-accumulators, hyperaccumulators have a higher expression of these oxidative stress response genes that confer enhanced tolerance to heavy metals. We predict that the mRNA expression patterns will parallel the induction or suppression of the protein enzymatic activities of the OSR genes in non-accumulators in response to heavy metal treatment, but mRNA expression of these OSR genes would be constitutively high in the hyperaccumulators regardless of treatment with heavy metals.

The central questions addressed in this thesis are: Is there a correlation between the expression of oxidative stress response genes and their respective enzymatic activity among both of these species, and if so, how strong is this correlation?

Significance of the Problem

mRNA expression analysis via Real-Time PCR can be used to compare expression levels of oxidative stress response genes in nickel-treated and control samples. These gene expression results can be compared with protein enzymatic activity and metal content to give some insight to the genetic mechanisms of heavy metal tolerance and accumulation in plants.

The species chosen for this study are of significance because of their membership in the *Brassicaceae* plant family. This family contains the most hyperaccumulator genera and species known, which gives researchers wishing to study the genetic component of hyperaccumulation a variety of plants for the engineering of a high-biomass, fast-growing hyperaccumulator (Prasad 2003). *Arabidopsis thaliana*, as a model organism, is also a popular research subject due to its' compact genome and ease of growth (Koornneef 2010).

CHAPTER II

MATERIALS AND METHODS

Plants used in Study and Seed Germination

The non-accumulator plant species *A. thaliana* (var. *Columbia*) seeds were obtained from LEHLE seeds (Round Rock, TX). The hyperaccumulator plant species: *T. montanum* var. *montanum* seeds were collected in the Wasatch Mountains of Utah by Ron Ratko of Northwest Native Seed (Seattle, WA).

Seeds were surface sterilized in a two step process. First 1 mL 70% Ethanol/0.02% Triton X-100 were added to the non-accumulator seeds in a 1.5 mL microfuge tube and the seeds let sit in this solution for 3 min (6 minutes for hyperaccumulator seeds). Next, the non-accumulator seeds were washed with 1 mL 50% bleach /0.02% Triton X-100 for 5 min. (10 min. for hyperaccumulator seeds). Both types of seeds were then washed 10 times with 1 mL sterile distilled water. For removal of excess water, seeds were placed on Whatman #3 filter paper disks, sterilized by either soaking with 100% ethanol or sterilized via autoclaving. Seeds were then placed onto ½ X MS (Murashige and Skoog) + 2% agar media in magenta boxes using sterile tweezers and sterile toothpicks. The seeds were allowed to germinate in the dark for 5 days. The magenta boxes were unwrapped and placed under a 9 hr. light cycle (125 μEinsteins light intensity) in the plant growth room at 23^{o}C and 60% RH for seeding growth to occur.

Hydroponics

After the seedlings had 2 true leaves, the magenta boxes were opened for 2 days to allow the seedlings to equilibrate to the humidity conditions present in the plant room.

A rectangular Styrofoam pad was constructed with 16 holes into which plants were inserted. A plastic microcentrifuge tube was cut at the 0.1 ml mark, a polyester plug inserted in the tube to hold the seedlings in place, and the seedlings placed in the Styrofoam pad holes. The plant roots were pulled through the tubes so they were exposed to the hydroponics solution but it was made sure that plant leaves were not in contact with the hydroponics solution. Then, the plants were transferred to black hydroponic tubs containing 8 liters of a 0.1 X Hoaglands nutrient solution (200 mL of 4 X Hoaglands solution, 40 mL of 40 mM FeDTPA, and 7760 mL distilled water). The nutrient solution was modified from the recipe from Hoagland and Arnon (1950) by the use of FeDTPA as indicated by Misra and Sharma (2006). The rims were covered with aluminum foil to prevent the entrance of light into the solution from the edges of the float in the tub, which was necessary to minimize the growth of algae. Air was bubbled into the hydroponics solution by using aerators to prevent anoxia.

Metal Treatment and Tissue Grinding

For the nickel treatment of plants, 2 mL of a 400 mM Nickel Acetate solution (100 μM Ni final concentration) was added to 8 L of hydroponics solutions (composed of 200 mL of 4 X Hoaglands solution, 40 mL of 40 mM FeDTPA, and 7760 mL distilled water). One tub of *Arabidopsis thaliana* and one tub of *Thlaspi montanum var. montanum* (10 plants in each tub) were treated for 12 days for each of the 3 experimental replicates. Control tubs of each species

had no nickel added and were grown under the same conditions as the treated plants. The plants were harvested and flash-frozen in liquid nitrogen, ground into a fine powder with a baked pestle in a baked mortar, and placed in 14 mL Falcon plastic tubes for storage at -80°C until the RNA isolation procedure and metal analysis (Into MS Thesis 2010).

Metal Analysis

After the plant tissue was ground and frozen, it was analyzed for nickel content using inductively coupled plasma- atomic emission spectroscopy (ICP-AES), or atomic absorption (AA) spectroscopy. The tissue samples were weighed out into 3 replicates (0.1-0.5 grams) and transferred to 100 mL beakers. Five mL nitric acid was added to each beaker and heated to 140ºC, and gently agitated until solution was reduced to a small volume (< 1 mL). After the samples were cooled, they were diluted 25 mL of deionized water. Fifteen mL of the digested plant samples were transferred into a 15 mL polypropylene centrifuge tube prior to running the samples on the ICP-AES or AA machine. The ICP-AES and AA machines were calibrated by using known analytical standards containing varying nickel concentrations. The mg/L nickel concentration was determined and multiplied by 0.025 (L) and divided by 1000, and then divided by the weight of the sample in grams to obtain the nickel concentration in ppm (Into MS Thesis 2010).

Primer Design

Degenerate primers for the SOD Cu/Zn, SOD Fe, SOD Mn, Catalase, and Glutathione Reductase genes were created by first determining the protein and DNA sequences for each of

the genes in the various *Brassicaceae* plant species present the NCBI database. Conserved regions among the genes sequences were determined to be useful as sequences for the constructing the primers. The potential primer sequences were run through the Primer Select software which is part of the Lasergene DNA Star software package. Primer sequences that had low T_m values for primer dimer and hairpin formation were ultimately chosen as primers to be used in this study.

Enzyme	5’ Protein Sequence	5’ Consensus Sequence	Tm (ºC)	3’ Protein Sequence	3’ Consensus Sequence	Tm (ºC)	Distance Apart in Nucleotide
Gluthathione Reductase	NLTPVA	AAY YTK ACW CCW GTB GCS	48.7	EEFVTM	CAT WGT BAC RAA YTC YTC	43.0	420
Catalase	VGNNFPVFF	GTY GGR AAC AAC TTY CCT GTH TTC TTC	60.7	NKNIDNFF	AAA GAA GTT RTC RAT GTT YTT GTT	56.2	543
SOD Cu/Zn	GDTTNGC	GGT GAY ACM ACW AAW GGM	44.2	TGNAGG/S	CSM RCC TGC GTT TCC MGT	53.7	276
SOD Fe	HWGKHH	CAY TGG GGM AAR CAY CAY	50.3	NHEFFWE	CTC CCA GAA GAA ATC ATG GTT	52.0	180
SOD Mn	HQKHHQ	CAY CAG AAR CAY CAY CAS	36.2	VINWKYA	TGC ATA TTT CCA GTT GAT YAC	52.5	501
G3PDH	FGIVEG	TTT GGM ATT GTT GAG GG	49.1	YDNEWG	ACC CCA TTC GTT GTC GTA	50.6	459

Table 1. *Brassicaceae* species degenerate primers for select enzymes.

RNA Isolation and DNAse Treatment of RNA

Guanidine Isothiocyanate total RNA Isolation Procedure

Frozen and ground plant tissue was added to 5 mL of GUISCN buffer in a 15 mL falcon 2059 polypropylene tube and mixed so that the total amount of tissue and buffer was a total of 7 mL in the tube. Seven Hundred µL ($1/10^{th}$ of total volume) of 2M sodium acetate (pH= 4) was added and mixed. Six mL of premixed phenol:chloroform 5:1 (5 mL of water saturated phenol and 1mL of chloroform) was added to the tube, mixed by inversion, and spun at 6,000 xg for 15 minutes at 4°C. The resulting upper phase was pipetted into a new falcon 2059 tube (~5mL). An equivalent amount of chilled (4ºC) isopropanol was added and the tube mixed by inversion. The sample was spun again at 6,000 xg for 10 minutes at 4°C. The supernatant was decanted off the top of the pellet. Two mL of TE + 0.5% SDS was added and mixed well so as to resuspend the pellet. Two mL of chloroform was added, the tube mixed by inversion, and spun at 6,000 xg for 10 minutes at 4ºC. The upper phase was removed and placed in a new falcon 2059 tube. Two hundred µL of 2M sodium acetate (pH = 5) (or 1/10 volume of the supernate) and 2 mL of isopropanol (or equal volume to the supernate) was added and the tube mixed by inversion. The sample was spun at 6,000 xg for 15 minutes at 4°C. The supernatant was decanted and 500 µL of 70% ethanol was added. The sample was spun again at 6,000 xg for 5 minutes at 4ºC. Then the supernatant was decanted and allowed to dry inverted on KimWipe for 15 minutes. Approximately 50-300 µL of DEPC treated double-distilled water was added (enough to completely dissolve product) and the sample was stored at -80°C.

DNAse Treatment of Total RNA

One tenth of the sample volume of RQ1 DNAse Buffer was added to the total RNA and mixed by pipetting. One twentieth of the sample volume of RQ1 DNAse enzyme was added to

the total RNA and mixed by pipetting. One one hundreth of the sample volume of RNAsin was added to the total RNA and mixed by pipetting. The sample was incubated at 37ºC for one hour and then 1/10th of the sample volume of RQ1 DNAse Stop solution was added to the total RNA and incubated at 65°C for 10 minutes. DEPC water was added to the tube to bring up the volume to 250 µL. Two hundred and fifty µL of phenol:chloroform (25:24:1) was added to the DNase treated total RNA, mixed, and microfuged at max speed (15,000 xg) for 10 minutes. The supernatant was pipetted into a fresh, 1.5 mL tube. One tenth of the sample volume of 2M NaOAc (pH = 5) was added to the tube. The tube was then filled to the top (1.5 mL mark) with isopropanol and placed in the -80ºC freezer for 30 minutes or until the solution was slushy. The sample was microfuged at max speed (15,000 xg) for 15 minutes. The supernatant was decanted and the pellet washed with 500 µL of 70% ethanol. The DNAse treated total RNA was spun again at max speed (15,000 xg) for 10 minutes. The supernatant was decanted and the pellet allowed to dry for 5-10 minutes. The DNAse treated total RNA was resuspended in 100-150 µL of DEPC water, or enough for the pellet to completely dissolve. The total RNA was then quantified using Nanodrop spectrophotometer.

PCR

First Strand Synthesis with AMV Reverse Transcriptase

Complimentary DNA was synthesized from DNase-treated total RNA (control and nickel-treated) from *Arabidopsis thaliana* and *Thlaspi montanum var. montanum* using each oxidative stress enzyme primer pairs. In addition, four cDNA synthesis reactions were set up for each species (control and nickel-treated) using G3PDH primers. This Real-Time PCR reaction

was used as a reference for the real-time polymerase chain reactions from the control and nickel treated plants amplified with gene specific primers. In each 0.2 mL PCR tube, approximately 1-3 µL of DNAse-treated total RNA template (600 ng DNAse treated total RNA), 2 µL 3' primer (50 pmol/µL), 4 µL PCR nucleotides (10 mM) and nuclease-free water was added up to a volume of 16 µL. The reaction was heated for 3 minutes at 65ºC to denature the RNA. Then 2 µL of 5X RT buffer, 1µL of RNase inhibitor, and 1 µL of AMV Reverse Transcriptase(10 U/µL) were added. The reaction was incubated at 45ºC for one hour. The AMV Reverse Transcriptase enzyme was inactivated by heating for 5 minutes at 90ºC. The resulting cDNA template was quantified via the use of a Nanodrop spectrophotometer and used for traditional polymerase chain reaction and real-time polymerase chain reaction.

Traditional PCR

Six PCR reactions were run for each species (oxidative stress enzymes, plus reference gene G3PDH), using the cDNA prepared from control and treated DNAse treated total RNA for a total of 24 reactions. In each 0.2 mL tube, approximately 100 ng of cDNA template, 25 µL of PCR mastermix, 1 µL of 5' primer (50 pmol/µL), 1 µL of 3' primer (50 pmol/µL), 1 µL of Taq (5 U/µL) were added and each tube then filled with nuclease-free water up to a total volume of 50 µL per reaction. Each reaction was run on BioRad iCycler gradient according to optimal primer annealing temperatures as indicated on table 1. The products were run on a 1% agarose gel to conform the presence of amplicons.

OSR and G3PDH gene fragment cloning and DNA Sequencing

Gene fragment cloning was performed on OSR genes and G3PDH in order to confirm the amplification of target genes. This was executed using the TOPO® TA Cloning® Kit by Invitrogen. DNA sequencing and BLAST searches of the clones confirmed the amplification of target genes.

Real –Time Polymerase Chain Reaction

Before performing Real-Time PCR on the cDNA product from the RT reactions, the concentration of the cDNA products was determined via Nanodrop spectrophotometry. This was done to ensure an optimal concentration of cDNA for the Real-Time PCR reaction. Once the optimal cDNA concentration was determined, a 10-fold serial dilution of template cDNA was employed for each species (control or treated) using primers for each gene of interest, including the reference gene. Three 10-fold dilutions of the cDNA template (0.1-10 ng cDNA total) were analyzed for each species using one of the primer pairs for each gene. This was done in triplicate to ensure data quality control (Yuan *et al*, 2006).

Once the cDNA was diluted to the optimal concentration, 5 µL of SYBR Green Supermix, 0.2 µL 3' primer (50 pmol/ µL), 0.2 µL 5' primer (50 pmol/ µL), 3.6 µL of sterile water, and 1 µL of 10-fold diluted template cDNA was added to each well in PCR plate array for a total of 10 µL per reaction. The reaction set up for all of the Real Time Reactions is summarized in the example table below.

Species/Treatment cDNA	Gene (*ex.*, SOD Mn)			
A. thaliana (Control)	Dilution Triplicate			
	0.1 ng	1.0 ng	10 ng	Sample
	0.1 ng	1.0 ng	10 ng	Triplicate
	0.1 ng	1.0 ng	10 ng	

Table 2. Example sample layout for Real-Time PCR plate array.

Cycle	Repeat	Step	Dwell Time (min)	Temperature (ºC)
1	1	1	2:00	95
2	40	1	0:15	95
		2 (annealing)	0:30	(varied)
		3	0:30	72
		4 (primer-dimer melting)	0:10	(varied)
3	1	1	0:30	72
4	1	1	1:00	55
5 (melt-curve)	80	1	0:10	55

Table 3. Example Cycle conditions for Real-Time PCR.

Values for cycles 2-2 and 2-4 depended on the inherent properties of the primers. Cycle 2-2 temperatures were determined upon the design of the primers (annealing temperatures), while cycle 2-4 temperatures were determined by melt-curve data using negative controls (SYBR Green, 3' and 5' primers, water, but no template). This value was typically 2-4 ºC higher than cycle 2-2 values.

CHAPTER III

RESULTS

Species/Treatment	Concentration in ppm (parts per million)
A.thaliana (Control)	128.41 ± 72.88
A.thaliana (Ni treated)	524.01 ± 92.04
T. montanum. var. montanum (Control)	108.01 ± 48.95
T. montanum. var. montanum (Ni treated)	1333.64 ± 161.43

Table 4. Nickel analysis of leaves performed using ICP-AES (YE Cerino unpublished results).

	TBARS (pmol/mg tissue)	PCARB (pmol/μg protein)
A.thaliana		
Control	5.45 ± 3.25	1.13 ± 0.66
Ni treated	80.04 ± 54.70 (p=0.000024)	1.72 ± 1.03 (p=0.07)
T. montanum. var. montanum		
Control	5.90 ± 2.63	1.28 ± 0.53
Ni treated	5.01 ± 2.56 (p=0.38)	1.24 ± 0.50 (p=0.82)

Table 5. Stress assay results for lipid peroxidation (TBARS) and protein carbonyls (PCARB) (Into MS Thesis 2010).

	SOD (mU SOD/μg protein)	CAT (μU CAT/μg protein)	GR (mU GR/μg protein)
A.thaliana			
Control	6.96 ± 2.78	409.52 ± 214.21	9.66 ± 5.55
Ni treated	13.28 ± 4.24 ($p=4.5 \times 10^{-5}$)	25.63 ± 47.48 ($p=2.32 \times 10^{-7}$)	31.83 ± 9.60 ($p=1.9 \times 10^{-8}$)
T. montanum. var. montanum			
Control	11.38 ± 10.25	3104.36 ± 1296.73	16.89 ± 10.04
Ni treated	19.44 ± 11.25 (p=0.049)	2586.93 ± 495.90 (p=0.16)	20.37 ± 11.05 (p=0.21)

Table 6. Protein activity levels in control and nickel-treated samples (Into MS Thesis 2010).

Relative expression levels in:	SOD Cu/Zn	SOD Fe	SOD Mn	CAT	GR
A.thaliana					
Control	1 ± 0.089	1 ± 0.073	1 ± 0.103	1 ± 0.111	1 ± 0.130
Ni treated	0.827 ± 0.082	0.860 ± 0.061	1.002 ± 0.081	0.94 ± 0.084	0.874 ± 0.130
T. montanum. var. montanum					
Control	1 ± 0.070	1 ± 0.112	1 ± 0.065	1 ± 0.087	1 ± 0.077
Ni treated	1.221 ± 0.087	1.109 ± 0.071	1.16 ± 0.105	1.04 ± 0.064	1.16 ± 0.063

Table 7. Real-time PCR relative comparison in individual species. *A. thaliana* control relative expression is set to 1 for comparison within species.

Relative expression levels in:	SOD Cu/Zn	SOD Fe	SOD Mn	CAT	GR
A.thaliana Control	1 ± 0.089	1 ± 0.073	1 ± 0.103	1 ± 0.111	1 ± 0.130
A.thaliana Ni treated compared to *A. thaliana* control	0.827 ± 0.082	0.860 ± 0.061	1.002 ± 0.081	0.94 ± 0.084	0.874 ± 0.130
T. montanum. var. montanum control compared to *A. thaliana* control	0.692 ± 0.058	0.675 ± 0.091	0.867 ± 0.067	0.890 ± 0.094	0.863 ± 0.076
T. montanum. var. montanum. Ni treated compared to *A. thaliana* control	0.846 ± 0.075	0.749 ± 0.054	1.004 ± 0.106	0.999 ± 0.061	1.004 ± 0.062
T. montanum var. montanum nickel treated compared to *A. thaliana* nickel treated	1.023 ± 0.082	0.871 ± 0.058	1.002 ± 0.098	1.057 ± 0.059	1.149 ± 0.067

Table 8. Real-time PCR relative comparison between treatments. *A. thaliana* control set to 1.

Gene	SOD Cu/Zn	SOD Mn	SOD Fe	Catalase	Gluthione Reductase	G3PDH
Accession	AAC24833.1	*	AAC24834.1	NM001036714.5	NP051065.1	NM001084061.1
Description	copper/zinc superoxide dismutase [*A. thaliana*]	*	iron superoxide dismutase 3 [*A. thaliana*]	catalase 2 (CAT2) mRNA, complete cds [*A. thaliana*]	*ATP synthase CF1 epsilon subunit [*A. thaliana*]	glyceraldehyde 3-phosphate dehydrogenase (GAPC2) [*A. thaliana*]
E value	1e-65	*	5e-40	0	2e-58	0.0
Max identity	98%	*	95%	99%	100%	98%

Table 9. BLAST hits of DNA sequences of clones. * indicates clone results were inconclusive.

As shown in Table 4, the Nickel concentration in *A. thaliana* control shoots was 128.41± 72.88, which was comparable to *T. montanum var. montanum* control shoots, which had a value of 108.01 ± 48.95 ppm. In nickel treated plants, *A. thaliana* was found to have a nickel concentration of 524.01 ± 92.04 ppm as compared to *T. montanum var. montanum*, which had a nickel concentration of 1333.64 ± 161.43 ppm.

Table 5 shows that *A. thaliana* control had a TBARS value of 80.04 ± 54.70 pmol/mg tissue and a PCARB value of 1.72 ± 1.03 pmol/μg protein. Nickel treated *A. thaliana* was found to have a significant increase in TBARS activity (p=0.000024). There was a slight, but not significant, increase (p=0.07) in PCARB activity in nickel treated *A. thaliana*. For *T. montanum var. montanum* control samples, it was found that TBARS level was 5.90 ± 2.63 pmol/mg tissue and the PCARB value was 1.28 ± 0.53 pmol/μg protein. The nickel treated samples had a PCARB value of 1.24 ± 0.50 pmol/μg protein and TBARS value of 5.01 ± 2.56 pmol/mg tissue,

which was not significantly different than their control counterparts (p=0.82 and p=0.38, respectively).

Upon examination of the data in Table 6, nickel treated *A. thaliana* samples were found to have a significant increase over control *A. thaliana* samples among SOD ($p=4.5 \times 10^{-5}$ and GR ($p=1.9 \times 10^{-8}$), while *A. thaliana* nickel treated samples showed a significant decrease in CAT ($p=2.32 \times 10^{-7}$). There was a significant increase in SOD activity in nickel treated *T. montanum var. montanum* (p=0.049), while control *T. montanum var. montanum* samples showed a slight, but not significant, increase in CAT (p=0.16) and GR (p=0.21) over their nickel treated counterparts.

Table 7 shows that, as compared to *A. thaliana* controls, there was a slight decrease of SOD Cu/Zn, SOD Fe, CAT and GR expression in *A. thaliana* nickel treated samples (relative values of 0.827 ± 0.082, 0.860 ± 0.061, 0.94 ± 0.084, and 0.874 ± 0.130, respectively). SOD Mn expression in Nickel treated *A. thaliana* showed a slight increase (relative value of 1.002 ± 0.081). There was an expression increase in nickel treated *T. montanum var. montanum* for all genes as compared to the *T. montanum var. montanum* control. The relative expression values of SOD Cu/Zn, SOD Fe, SOD Mn, CAT and GR were 1.221 ± 0.087, 1.109 ± 0.071, 1.16 ± 0.105, 1.04 ± 0.064, and 1.16 ± 0.063, respectively.

Table 8 indicates that, compared to *A. thaliana* control plants, base level of relative expression of SOD Cu/Zn, SOD Fe, SOD Mn, CAT, and GR in *A. thaliana* nickel treated plants were 0.827 ± 0.082, 0.860 ± 0.061, 1.002 ± 0.081, 0.94 ± 0.084, and 0.874 ± 0.130, respectively. Compared to the *A. thaliana* control plants, the base level of expression for SOD Cu/Zn, SOD Fe, SOD Mn, CAT, and GR in *T. montanum var. montanum* control plants was 0.692 ± 0.058,

0.675 ± 0.091, 0.867 ± 0.067, 0.89 ± 0.094, and 0.863 ± 0.076, respectively. In comparison to *A. thaliana* control plants, the base level of expression for SOD Cu/Zn, SOD Fe, SOD Mn, CAT, and GR in *T. montanum var. montanum* nickel treated plants was 0.846 ± 0.075, 0.749 ± 0.054, 1.004 ± 0.106, 0.999 ± 0.061, and 1.004 ± 0.106, respectively. Finally, in comparison to *A. thaliana* nickel treated plants, the base level expression of SOD Cu/Zn, SOD Fe, SOD Mn, CAT, and GR in *T. montanum var. montanum* samples nickel treated plants was 1.023 ± 0.023, 0.871 ± 0.058, 1.002 ± 0.098, 1.057 ± 0.059, and 1.149 ± 0.067, respectively.

As shown in Table 9, SOD Cu/Zn, SOD Fe, CAT, and G3PDH were found to correspond to expected BLAST hits upon inquiry. The BLAST hit for the SOD Cu/Zn PCR fragment corresponded with the expected product upon inquiry with an Error value of 1e-65 and a maximum identity of 98%. No successful clone could be isolated for SOD Mn; as a result, no sequencing data or BLAST inquiry could be done. The BLAST hit for SOD Fe showed that the expected product upon inquiry was isolated with an Error value of 5e-40 and a maximum identity of 95%. The BLAST hit for CAT confirmed that expected product was produced and had an Error value of 0% and a maximum identity of 99%. BLAST hit for GR showed different gene than expected which could be due to mis-cloning of a secondary PCR product or sequencing errors by sequencing the wrong clone. The BLAST hit for G3PDH gave the expected product upon inquiry with an Error value of 0% and a maximum identity of 98%.

CHAPTER IV

DISCUSSION

The control *A. thaliana* and *T. montanum var. montanum* plants both contain about the same amount of nickel. This is an indication that the baseline stress levels should be comparable between the two plant species. The *T. montanum var. montanum* plants appear to accumulate more nickel than *A. thaliana* during nickel exposure, as the nickel amount in these plants was approximately 2.5x that of the nickel treated *A. thaliana* plants. As indicated by nickel content analysis in Table 4, *T. montanum var. montanum* plants contain more than the minimum amount of metal needed to qualify as a hyperaccumulator plant (1000 ppm for nickel). *A. thaliana* is confirmed as a non-accumulator due to it containing less than the 1000 ppm metal content threshold of hyperaccumulators (Boyd 1999*)*.

As shown in Table 5, *A. thaliana* plants are stressed by the presence of nickel as indicated by the TBARS assay. In nickel treated *A. thaliana* plants, the TBARS level was shown to be 16x higher than the control plants, which was a significant increase ($p=0.000024$). Stress by nickel treatment of *A. thaliana* plants was also slightly higher as indicated by the PCARB assay but the change indicated by this assay was not found to be significant ($p=0.07$).

In comparison to *A. thaliana*, The TBARS and PCARB values for control *T. montanum var. montanum* plants were comparable and not very different. This indicates that the baseline of

stress between the two plant species was similar. However, compared to the TBARS assay values of nickel treated *A. thaliana* plants, the TBARS level in nickel treated *T. montanum var. montanum* plants was 16x lower and comparable to the TBARS levels in control *T. montanum var. montanum* samples. This indicates that the *T. montanum var. montanum* plants were not stressed by nickel treatment unlike *A. thaliana* plants. The values of the PCARB assay for *T. montanum var. montanum* were also comparable to those *A. thaliana* in both nickel treated and control samples. Due to the fact that PCARB did not show stress indication for either species under nickel treatment, PCARB may not be the best assay for the analysis of nickel stress in these plants. Since TBARS showed a significant difference in *A. thaliana* nickel treated plants this assay seems to be a better test for stress by heavy metals in plants.

As shown in Table 6, there was an increase in the enzyme base activity (SOD and CAT) for both control and nickel treated *T. montanum var. montanum*, when compared to the base activity in *A. thaliana* control and nickel treated plants. However, GR did not show a marked increase. Upon nickel treatment, *A. thaliana* plants showed an increase in GR activity (Table 6) which was higher than the increase in GR activity shown in nickel treated *T. montanum var. montanum* plants. CAT levels in nickel treated *A. thaliana* was shown to be significantly lower than CAT levels in control *A. thaliana* ($p=2.32 \times 10^{-7}$). Nickel treated *T. montanum var. montanum* also showed slight, but insignificant, decrease in CAT levels when compared to control *T. montanum var. montanum* CAT levels.

Real-time PCR analysis of gene expression was performed to provide a relative comparison of the expression of the OSR genes between treatments and between species. Because SOD Mn and GR had inconclusive clone results, the relative expression levels of these genes may be indeterminate. As shown in Table 7, in comparison to the *A. thaliana* control

plants, the base level of expression of SOD Cu/Zn, SOD Fe, CAT, and GR in nickel treated *A. thaliana* plants was reduced by 17.3%, 14%, 6%, and 12.6%, respectively. However, the exception was the SOD Mn gene, which had an increase of expression of 0.2%.

When compared to the *A. thaliana* control plants (Table 8), the base level of expression for all OSR genes; SOD Cu/Zn, SOD Fe, SOD Mn, CAT, and GR in the control *T. montanum var. montanum* plants was found to be was lower by 30.8%, 32.5%, 13.3%, 11%, and 13.7%, respectively. Relative to the to *A. thaliana* control plants, the base level of expression for SOD Cu/Zn, SOD Fe, and CAT in nickel treated *T. montanum var. montanum* plants was shown to be reduced by 15.4%, 25.4%, and 0.1%, respectively. Expression of SOD Mn and GR was elevated in nickel treated *T. montanum var. montanum* plants by 0.4% and 0.4%, respectively as compared to *A. thaliana* control plants. Compared to *A. thaliana* nickel treated plants, the base level of expression of SOD Cu/Zn, SOD Mn, CAT, and GR in nickel treated *T. montanum var. montanum* plants were elevated by 2.3%, 0.2%, 5.7%, and 14.9%, respectively. Conversely, when compared to *A. thaliana* nickel treated plants, the SOD Fe expression levels in *T. montanum var. montanum* plants was decreased by 12.9%.

Conclusion

RNA expression of OSR genes in *A. thaliana* and *T. montanum var. montanum* does not vary much, as the maximum variability is 32.5% for SOD Fe in control *T. montanum var. montanum* when compared to control *A. thaliana* plants. In nickel treated *A. thaliana* plants, SOD Cu/Zn had the highest expression variability of 17.3% when compared to *A. thaliana* plants. In nickel treated *T. montanum var. montanum*, the highest expression variability was

25.4% for SOD Fe when compared to *A. thaliana* plants. When compared to *A. thaliana* nickel treated plants, the highest variability in nickel treated *T. montatnum var. montanum* was 14.9% for GR. Within the two species, gene expression varied, at the most, by 1.221 ± 0.087-fold in nickel treated *T. montanum var. montanum.* The highest fold variability in nickel treated *A. thaliana* was 1.002 ± 0.081 for SOD Mn. Among the two species gene expression varied, at the most, by 1.004 ± 0.106-fold and 1.004 ± 0.062-fold in nickel treated *T. montanum var. montanum* for SOD Mn and GR, respectively. In nickel treated *A. thaliana*, gene expression varied the most in SOD Mn by 1.002 ± 0.098-fold. In control *T. montanum var. montanum*, gene expression varied the most in CAT by 0.89 ± 0.094-fold. When nickel treated *T. montanum var. montanum* samples were compared with nickel treated *A. thaliana* samples, the highest expression variability occurred in GR, which had a 1.149 ± 0.067-fold increase.

The SOD Mn and GR data are somewhat unclear due to the fact that from the RT-PCR reactions we were unable to clone PCR fragments that corresponded to these genes. However, the cloning may have only resulted in the isolation of minor contaminant fragments and not the main PCR products as we did not screen enough clones and sequence them in order to find the main PCR products. The SOD Mn and GR data may still be valid in that upon analysis of the Real-Time PCR melt curves, there was found to be only one major product with a Tm of 88 ºC and 89 ºC respectively, and no apparent minor products resulting in different melt curves. Therefore based on the melt curves for these two amplified products, these results indicate that the major Real-Time PCR products being analyzed were in fact from the genes in question.

The central dogma of biology states that DNA codes for RNA, and RNA then codes for protein. However, there are regulatory steps in eukaryotes that modulate the levels of transcription, translation, and accumulation of functional proteins. Abundant amounts of mRNA

do not necessarily correlate to a high amount of protein or enzyme activity levels in a cell (Greenbaum *et al*, 2003), which seems to be the case in this particular study. At the level of transcriptional regulation, there appears to be no substantial difference among the level of expression of OSR genes with regard to nickel treatment or plant species. This observation probably means that the level of transcriptional regulation does not appear to be tied to the induction of OSR genes by nickel treatment, nor is it species specific. Regardless of treatment or particular plant species, the level of OSR gene expression appears to be rather constant. This results in a conundrum with regard to the level of the OSR enzyme activity when correlated to the metal content of the plants. It has been found that the nickel concentration and OSR enzyme activity is significantly higher in the hyperaccumulator plant *T. montanum var. montanum* as compared to the non-accumulator plant *A. thaliana* (Into MS Thesis 2010). Therefore, some other level of regulation may be affecting the observed outcome of increased OSR enzyme activity in the nickel treated plants. Other steps in the regulatory process such as mRNA stability, mRNA translation, post-translational protein modification or sensitivity of the enzymes to heavy metals may be the regulatory points influencing OSR enzyme activity.

One hypothesis which may explain this phenomenon is that the presence of nickel compromises the structural integrity of the OSR mRNAs, which may negatively affect the functionality of the mRNA during translation (Grunberg-Manago 1999). Another potential explanation is perhaps OSR mRNAs are more stabilized in hyperaccumulators and have longer half-lives for translation. By adding nickel (or any oxidative stressor), OSR mRNA stability might be compromised, especially when the oxidative stressor has a long-term, chronic influence on the plant (as was the case in this study) versus a brief exposure to a stressor (Abdelmohsen *et al*, 2008). Alternatively, oxidative stress caused by the presence of heavy metal could also affect

protein activity (Schutzendubel & Polle, 2002). In *T. montanum var. montanum*, perhaps the oxidative stress response enzymes are more stable when exposed to oxidative stress, thus more able to retain their activity. A final explanation may be that the OSR enzymes of hyperaccumulator plants are less sensitive to heavy metal substitutions in their active sites which may affect enzyme activity more than the OSR enzymes in non-accumulators. Therefore, OSR enzymes from hyperaccumulator plants may retain the correct metal center for enzymatic activity, whereas non-accumulator OSR enzymes may be susceptible to the wrong metal being substituted into the enzyme active site to inhibit enzymatic activity (Assche & Clijsters 2006).

However, to determine if hyperaccumulators have more translated oxidative stress response proteins upon nickel treatment than non-accumulators, western blot analysis is needed. Western blot analysis specifically tests the relative quantity of a target protein by immunodetection, in which a protein-specific antibody binds to the protein after proteins have been sorted out by their size using gel electrophoresis (Heidebrecht 2009). This assay can help determine the actual levels of translated OSR proteins present in a cell before and after nickel treatment. For the determination of nickel inhibition of species specific OSR enzymes an *in vitro* transcription-translation assay is needed to produce *in vitro* the OSR enzymes and assay their activity in the presence and absence of nickel to determine any inhibitory effects that this metal may have on OSR enzyme activity (Rosenblum *et al*, 2012).

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BIOGRAPHICAL SKETCH

Florestella Ruiz attended Veterans Memorial High School in 2000 and earned her high school diploma in 2004. She was later was accepted to The University of Texas-Pan American where she majored in biology and earned her Bachelor's Degree of Science in the spring of 2009. During her senior year as an undergraduate, she worked as a research and laboratory assistant in molecular biology under Dr. Michael Persans, who guided her into following a higher level of education in biology with a Master's degree. Following her graduation she was soon accepted to the graduate program in the summer 2009 for the Department of Biology at the University of Texas-Pan American under the University of Texas Medical Branch-Galveston Bridges to Ph.D. program. After defending her thesis in Spring 2012, focused on plant molecular biology, she earned her Master's Degree of Science in Biology. Her permanent place of residence is now in San Antonio, TX preparing for the next chapter in her life with her new degree.