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SfMTP1 and SpMTP1 Cloning and Expression in Saccharomyces Cerevisiae: Potential for The Genetic Engineering of Arabidopsis Thaliana

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SFMTP1 AND *SPMTP1* CLONING AND EXPRESSION IN *SACCHAROMYCES CEREVISIAE*: POTENTIAL FOR THE GENETIC ENGINEERING OF *ARABIDOPSIS THALIANA*

A Thesis

by

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ABSTRACT

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To evaluate metal tolerance conferred by the overexpression of Metal Tolerance Protein 1 (MTP1) in transgenic non-accumulator plants such as *Arabidopsis thaliana*, a model system was developed in *Saccharomyces cerevisiae* containing either *SfMTP1* cDNA, from the metal tolerant plant *Streptanthus farnsworthianus,* or *SpMTP1* cDNA, from the hyperaccumulator *Streptanthus polygaloides*. Nonquantitative RT-PCR showed constitutive expression of *MTP1* in both plant species. Alignment of SfMTP1 and SpMTP1 protein sequences shows a gap in SpMTP1 in a variable histidine region known to affect metal transport. Disk assays revealed *SfMTP1* cDNA imparted greater Ni tolerance to yeast than *SpMTP1* cDNA; however, *SpMTP1* showed higher Cd tolerance than *SfMTP1*. No significant difference was found with Co; but both cDNAs made yeast slightly more Zn sensitive. MTP1 histidine-rich regions control metal transport mechanisms, but differences in these regions may be responsible for higher tolerance of specific metals between different *Streptanthus* species.

DEDICATION

For my parents, who have supported me through the years and encouraged me to be stubborn and hardworking. I could not have completed my thesis without their support and confidence. I would also like to dedicate this to Alma Garza who always wants what is best for her family and could not be happier to know that I still want to continue my education. And finally, I could not have done this without Ediel Perez, who sat behind me and encourage me to keep writing.

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CHAPTER 1

INTRODUCTION

Growing in the serpentine soils of California are the jewelflower flora *Streptanthus farnsworthianus* and *Streptanthus polygaloides* (Reeves *et al.*, 1983; Calflora, 2013; CNPS, 2013). These plant species have an unusually higher metal tolerance and an efficient metal uptake mechanism as compared to non-accumulator plants. *S. farnsworthianus* and *S. polygaloides* are members of the Brassicaceae family, which also includes *Arabidopsis thaliana*, the model plant (Peer *et al.*, 2006). The Brassicaceae family contains 25% of currently known hyperaccumulators and has the largest amount of Ni hyperaccumulating taxa (Rascio and Navari-Izzo, 2011). In particular, the plant species *S. polygaloides* is an excellent Ni hyperaccumulator (Reeves *et al*., 1981; Reeves *et al*., 1983). The *Streptanthus* genera is listed among the most diverse genera for serpentine endemics and is known for having groups of taxa which rapidly speciates to generate varieties (Safford *et al*., 2005). This speciation of the *Streptanthus* genera may be the cause of the many different species that show differing metal accumulation abilities. *S. farnsworthianus* and *S. polygaloides* are noteworthy species in this genera because of their potential metal tolerance and uptake abilities. These species have not been thoroughly investigated and may yet provide new insight to metal accumulator and hyperaccumulator traits.

A hyperaccumulator is defined as a metal accumulating plant that concentrates more than 10 mg/g (1% of shoot dry mass) Mn or Zn, more than 1 mg/g (0.1% of shoot dry mass) As, Co, Cr, Cu, Ni, Pb, Sb, Se, or Tl, or more than 0.1 mg/g (0.01% of shoot dry mass) Cd in its aerial

tissues without suffering from the toxic effects of the heavy metals (Baker and Brooks, 1989; Reeves and Baker, 2000; Ingle *et al.*, 2005; Peer *et al.*, 2006; Rascio and Navari-Izzo, 2011). A comparison between a hyperaccumulator and non-accumulator plant species identifies key differences that allow hyperaccumulating plants to accumulate more metal. Hyperaccumulators are able to translocate metal ions from the root to shoots faster than a non-accumulator and they can more efficiently detoxify and compartmentalize metal contaminants into the vacuole (Rascio and Navari-Izzo, 2011).

Potential reasons that metal accumulation has evolved in hyperaccumulator plants is debatable and many hypotheses have been proposed. Hyperaccumulation may have developed as a response to reduce competition, escape pathogen transmission from other plants, or provide a defense against herbivory (Kay *et al*., 2011; Strauss and Boyd, 2011). Other suggested hypotheses are to maintain species survival when exposed to metal-releasing industries that pollute the environment (Whiting *et al.*, 2004) and the joint effects hypothesis (Strauss and Boyd, 2011; Rascio and Navari-Izzo, 2011). The joint effects hypothesis states that combinations of heavy metals accumulated by a plant will have an additive effect and cause aerial tissues to be more toxic to herbivores (Strauss and Boyd, 2011; Rascio and Navari-Izzo, 2011).

Hyperaccumulators are able to uptake and tolerate high amounts of heavy metals because they have evolved mechanisms to protect themselves from heavy metal stress. Mechanisms such as chelation, volatization, and vacuolar compartmentalization have evolved in hyperaccumulator plant species to enable these plants to sustain living in metal polluted soils (Wu *et* al., 2010). Chelation utilizes ligands such as histidine and citrate, to bind metal ions. Binding ions to the ligands causes the metal ions to lose their charge or become inert and thus reduce damage to the

plant cell (Wu *et al*, 2010). Volatization is used by some plants to convert metal ions into a volatile form and release these compounds into the air; notably mercury and the non-metal selenium (Lone *et al.*, 2008, Wu *et al.*, 2010). Vacuolar compartmentalization is the sequestration of metals into the vacuole thereby partitioning the metal ions and preventing them from damaging other areas of the plant cell (Wu *et* al., 2010; Kay *et al*., 2011). Even though some hyperaccumulator characteristics have been defined, the mechanisms that contribute towards the comprehensive metal hyperaccumulation characteristic require additional study.

The metal tolerance protein 1 (MTP1) is known to mediate the transport of metals across the cell and vacuolar membranes (Verbruggen *et* al., 2009; Rascio and Navari-Izzo, 2011). Differential expressions of common genes, such as MTP1, play essential roles in plant accumulation (Rascio and Narvari-Izzo, 2011). Different levels of expression of these genes affect metal regulation and account for different metal accumulating characteristics within a genus.

The MTP1 protein structure is made of six transmembrane domains formed with hydrophobic amino acids arranged in six alpha helices and contains a Cation Diffusion Facilitator (CDF) signature sequence common to only members of the CDF family (Paulsen and Saier, 1997). This is a significant characteristic because proteins of the CDF family are exclusively involved with the transport of heavy metal ions (Paulsen and Saier, 1997). Between the fourth and fifth cytoplasmic loops, exists a variable histidine region with the sequence $(HX)_n$ where X is usually G or D and n= 3-6 (Paulsen and Saier, 1997). This histidine rich region is significant because these histidine residues are known to interact with metals and therefore may influence metal transport (Yamashita *et al*., 1990; Ingle *et al.*, 2005). Further investigation of the

duplications within the variable histidine region of the *MTP1* gene is necessary to understand how the repetitive sequences can influence regulation and transport of metals.

Statement of the Problem

Increased amounts of heavy metal contaminants in soil and water are expected to rise in industrial, urban, and agricultural land from activities such as mining, transport, and waste disposal (Kärenlampi *et al.*, 2000; Jarup, 2003; Duruibe *et al.*, 2007). Plants accumulate heavy metals in soil; thereby entering the food chain and harming organisms at all trophic levels. This is a serious problem, because heavy metal pollution causes kidney damage, low bone density, cardiovascular diseases, lung disease, damage to the nervous system, and many other varying health related symptoms (Jarup, 2003, Duruibe *et al.*, 2007, Lone *et al*., 2008). Of greater concern are heavy metals that do not biologically or chemically degrade which makes them difficult to remove from the organism or environment (Duruibe *et al*., 2007; Wu *et al.*, 2010).

Phytoremediation is a popular method to bioremediate contaminated soils (Lone *et al*., 2008). Phytoremediation is the use of plants to clean up polluted soils and water (Umeoguaju, 2009; Wu *et al.*, 2010). This method uses a plant's natural ability biofilter and phytoextract contaminants from soils. Phytoremediation has recently gained popularity, because it is environmentally friendly, money-saving, and provides a pleasing appearance during the decontamination stages. Additionally, metals can be recycled from harvested stems and leaves to prevent re-integration of metals into the soil (Wu *et al*., 2010). Phytomining or the recycling of metals from harvested stem and leaves of a plant increases the financial benefit of this method.

Ideally, natural growing hyperaccumulator plants can be used for phytoremediation, however, most natural hyperaccumulators tend to grow slow and have a low biomass-production

(Kärenlampi *et al.*, 2000; Wu *et al.*, 2010). It is not practical to use these plants, because they would take a long time to phytoextract toxic metals from the soils. Additionally, the smaller plant mass results in less accumulated metal removed from the soil. An ideal phytoremediation plant should have the following qualities: a high biomass-production, fast growth, a high metal tolerance, and an efficient metal accumulating ability (Kärenlampi *et al*., 2000; Clemens *et al*., 2002). Efforts to produce a more cost efficient and safer environment alternative have promoted genetic engineering research to produce a phytoremediation plant with these qualities. Utilizing modern molecular and genetic techniques, a non-accumulator plant can be engineered to acquire metal tolerance and uptake mechanisms conveyed by hyperaccumulator genes. To quickly evaluate engineered metal tolerance in an organism, accumulator and hyperaccumulator metal tolerance genes can be transferred into metal sensitive yeast and assayed for the metal tolerance parameters conveyed by the gene (Persans *et al.* 2001; Kim *et al*., 2004).

Conceptual Framework

Metal tolerance conferred by MTP1, from *S. farnsworthianus* and *S. polygaloides*, was investigated in yeast and genetically engineered plants were produced for future analysis. Total RNA extracted from the shoot tissue of the accumulator *Streptanthus farnsworthianus* and hyperaccumulator *Streptanthus polygaloides* were used as a template to produce *MTP1* cDNAs through RT-PCR. *MTP1* cDNAs were inserted into the *Escherichia coli*/*Saccharomyces cerevisiae* shuttle vector *pYES2* and *Agrobacterium tumefaciens*/plant transformation vector *pKYLX71*. These genes were confirmed to be present in the vectors through DNA sequencing before being used to transform a metal sensitive yeast strain of *Saccharomyces cerevisiae* (*Δpep5::trp1*) and *Arabidopsis thaliana*. Yeast containing an empty vector *pYES2* (control), accumulator *SfMTP1*, and hyperaccumulator *SpMTP1* were assayed for metal tolerance by

exposure to nickel, zinc, cobalt, and cadmium. *A. thaliana* flowers were dipped in an *Agrobacterium* media containing transformed *SpMTP1* gene to produce genetically modified seeds. Seeds collected from the transformed *A. thaliana* flowers were germinated on kanamycin selection plates to select for plants that contained the *SpMTP1* transgene. Transgenic *A. thaliana* will be grown until the third generation (T_3) before being investigated for their metal accumulation properties. The goal of this study is to determine the differences in metal tolerance that the *S. farnsworthianus* and *S. polygaloides* MTP1s confer to metal sensitive yeast, and to grow transgenic *A. thaliana* overexpressing these MTP1s for future studies. These species were chosen because they are model metal accumulating plants that have characteristics that are desirable for the genetic engineering of an ideal phytoremediation plant.

Statement of the Purpose

The purpose of this thesis is to create transgenic yeast and plant models for the study of metal tolerance conferred by accumulator and hyperaccumulator *MTP1*s. The yeast metal tolerance assays were designed to test the ability of the plant *MTP1*s to impart nickel, cobalt, zinc, or cadmium tolerance to metal sensitive yeast. The hypothesis is that if an accumulator and hyperaccumulator *MTP1* gene are transformed into yeast, the transgenic yeast will have increased metal tolerance. In addition, the yeast transformed with hyperaccumulator *SpMTP1* should show a higher metal tolerance than the accumulator *SpMTP1* for each metal the modified yeast are exposed to. Data derived from the yeast assays allows exploration of differential *MTP1* regulation of metal ion uptake. Furthermore, MTP1 sequencing results from this study can be compared to other Brassicaceae plant species MTP1 proteins for further insight into the role of MTP1 and how the histidine rich region regulates metal transport. Overall, this study can also be

used to aid in determining the best Brassicaceae hyperaccumulator genes for use in future genetic manipulation to produce an ideal phytoremediation plant.

CHAPTER II

MATERIALS AND METHODS

Plant Collection and Maintenance

Seed Collection and Storage

The seeds used in this experiment were from the Brassicaceae plant species *Streptanthus polygaloides, Streptanthus farnsworthianus,* and *Arabidopsis thaliana* variety *columbia*. Non-accumulator *A. thaliana* plants were grown from seed stocks derived from the LEHLE seeds of Round Rock, Texas. The accumulator plant species, *S. farnsworthianus,* were grown from seed stocks previously collected from the Sierra Nevada Mountains in California by Mr. Ron Ratko of Northwest Native Seed (Seattle, WA). Hyperaccumulator *S. polygaloides* plants were grown from seeds previously collected from the Sierra Nevada Mountains in California by Dr. Robert Boyd of Auburn University. All seeds were stored in 1.5 mL microfuge tubes at room temperature, containing a large desiccation rock, until ready for sterilization.

Seed Sterilization

Non-accumulator. In a laminar flow hood, about 50 µl of *A. thaliana* seeds were put into a sterile 1.5 mL microfuge tube in preparation for surface sterilization. One mL of 70% Ethanol/0.02% Triton X-100 was used to wash the seeds. The seeds were allowed to settle for 3 minutes before pipetting out the ethanol/Triton X-100 mixture. One mL of 50% bleach/0.02% Triton X-100 was added to the non-accumulator seeds and mixed by inverting the tube several

times. The seeds were allowed to sit for 5 minutes with a sporadic mix at 2 minutes. The bleach mixture was removed and the seeds were rinsed 10 times with 1 mL of sterile ddH_2O. Whatman #3 (90 mm diameter) filter paper was sterilized by soaking the paper in 100% ethanol and air drying in the laminar flow hood. Using an autoclaved toothpick, the seeds were moved to sterile Whatman #3 filter paper to air dry in the laminar flow hood. Once the seeds were dry, they were then plated on ½ X MS (Murashige and Skoog) + 1 % agar media in 150 mm x 10 mm petri dishes, parafilmed, and covered with aluminum foil. *A. thaliana* seeds were vernalized for 5 days in the dark at 4°C. Subsequently, the petri dishes were moved to the plant growth room, unwrapped, and placed under a 9 hour light cycle (125 µEinsteins light intensity). The seeds were germinated at 23°C and 60% RH (relative humidity).

Accumulator and Hyperaccumulator. In a laminar flow hood, 100 µl of seeds were put into a sterile 1.5 mL microcentrifuge tube and surface sterilized with 1 mL of 70% Ethanol/0.02% Triton X-100. The seeds were allowed to sit for 3 minutes for *S. farnsworthianus* or 6 minutes for *S. polygaloides* and then the ethanol/Triton X-100 mixture was removed. One mL of 50% bleach/0.02% Triton X-100 was added to the seeds and the tube was allowed to sit for 5 minutes for the accumulator plant species or 10 minutes for hyperaccumulator plant species. After removal of the bleach mixture, the seeds were rinsed 10 times with 1 mL of sterile ddH_2O. The seeds were moved to sterile Whatman #3 filter paper using sterile toothpicks or sterile tweezers and allowed to air dry. To stimulate seed germination, the seeds were moved to aluminum wrapped magenta boxes containing ½ X MS media + 2% agar and placed at room temperature for 5 days for *S. farnsworthianus* or at 4°C for 7 days for *S. polygaloides*. Once the required time had passed, the magenta boxes were unwrapped and moved to the plant growth

room and placed under a 9 hour light cycle (125 µEinsteins light intensity). Seed germination and plant growth occurred at a temperature of 23°C and 60% RH (Into MS Thesis, 2010).

Hydroponics

Once the plants had sprouted 2 true leaves, the petri dish was opened and the *A. thaliana* plants were allowed to equilibrate to the humidity of the plant growth room overnight. *S. farnsworthianus and S. polygaloides* plants were allowed to acclimatize to the plant growth room humidity for 2 days. The seedlings were next moved to specially designed hydroponics apparatus for further development and growth. The hydroponics system was composed of a 10 liter black tub, a black rectangular Styrofoam float, and an air pump. The Styrofoam float had 16 holes, punctured into it, that were large enough to fit a 1.5 mL microcentrifuge tube. When the seedlings were ready for transfer, they were carefully placed into a plastic microcentrifuge tube that had been cut at the 0.5 mL mark and the plants held in place with a polyester fabric plug. The tubes were fitted into the Styrofoam float with the roots touching the Hoagland solution beneath the float and the leaves just above the surface. Two hydroponics tubs were made for each plant species. The hydroponics tubs contained 8 liters of 1/10 X Hoaglands nutrient solution. The solution used was modified from the original recipe provided from Hoagland and Arnon (1950) with the addition of FeDTPA (Misra and Sharma, 2006). The final plant growth nutrient solution contained 200 mL of 4 X Hoaglands solution, 40 mL of 40 mM FeDTPA, and 7760 mL distilled water. In order to prevent algae growth, aluminum foil was used to cover the gaps in between the Styrofoam pad and the black tub rims that were exposed to light. A 10 L-30 L fish tank pump aerator was used to bubble air into the hydroponics tub. A plastic tube, connected to the aerator, was inserted into one of the 16 holes in the Styrofoam pad and air was bubbled into the solution to prevent anoxic conditions.

Tissue Storage

Plants were grown for 2 to 3 months in the hydroponics system or until they appeared bushy. Once the plants matured, each species was separately taken to the lab and the shoots were separately flash frozen with liquid nitrogen. Using a sterile pestle, the hardened plant tissue was ground to a fine powder in a sterile mortar. With a baked spatula, the resulting tissue powder was transferred into a 50 mL centrifuge tube and stored at -80°C for later use.

Total RNA Isolation

Guanidine Isothiocyanate/Phenol Chloroform Total RNA Extraction

Powdered plant tissue, for each plant species, was individually transferred into 15 mL tubes containing 5 mL of GUISCN (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroyl sarkosine/ 0.72% β-Mercapto-ethanol, pH 7) buffer. The amount of tissue plus GUISCN buffer did not total over 7 mL per single 15 mL tube. One tenth the volume of 2 M sodium acetate (pH 4.0) was added to the tube and mixed. Five mL of phenol/chloroform (5:1) was added to the solution and mixed until a milky white appearance was seen. The tubes were spun at 6000 xg for 15 minutes at 4°C. Then, in a new 15 mL tube, the supernatant was combined with 7 mL of isopropanol, mixed well, and spun for 10 minutes at 6,000 xg at 4°C. The resulting pellet was resuspended in 4 mL of 4 M lithium chloride and spun for 10 minutes at 6,000 xg at 4°C. After decanting the supernatant, the pellet was resuspended in 2 mL of TE + 0.5% SDS and 2 mL of chloroform. The sample was mixed until it appeared milky white and spun for another 10 minutes at 6,000 xg at 4°C. Next, the upper phase was transferred to a new 15 mL tube and combined with one tenth volume 2 M sodium acetate (pH 5.0) and an equal volume of isopropanol. This solution was spun for 15 minutes at 6,000 xg at 4°C. The resulting pellet was resuspended in 500 µL of 70% ethanol and spun for 5 minutes at 4°C . After air

drying for 15 minutes, the pellet was resuspended in 50 to 300 µL of DEPC ddH_2O. All total RNA samples were stored at -80°C.

DNAse Treatment of total RNA

In a new 1.5 mL microcentrifuge tube, 100 µL of total RNA, 68 µL DEPC ddH_2O, one-hundredth volume RNAsin, one tenth volume RQ1 DNAse Buffer, and one-twentieth volume RQ1 DNAse enzyme (1 U/µL) (Promega, Madison, WI) were combined to total a final solution of 200 µL. The tube was then incubated in a water bath at 37°C for 1 hour. Next, one tenth volume of RQ1 DNAse Stop solution was mixed into the tube and the microcentrifuge tube was incubated at 65°C for 10 minutes on a heat block. After heat denaturation of the DNAse enzyme, 30 µL of DEPC ddH_2O was added to the tube to bring the volume up to 250 µL. An equal volume of 250 µL phenol/chloroform/Isoamyl alcohol (25:24:1) was added to the microcentrifuge tube, mixed well, and spun for 10 minutes at 14,000 xg. The supernatant was removed to a new 1.5 mL microcentrifuge tube and one tenth volume of 2 M sodium acetate (pH 5.0) was added. To pellet the RNA, about 1,200 µL of isopropanol was mixed into the solution and then the tube was chilled at -80°C for 30 minutes until slushy. The microcentrifuge tube was spun for 15 minutes at 14,000 xg. The supernatant was removed and the pellet was washed with 500 µL ethanol and spun for 5 minutes at 14,000 xg. The supernatant was pipetted off and the pellet was air dried for 15 minutes and resuspended in 100 to 150 µL of DEPC ddH_2O. The total RNA was quantified using a Nanodrop spectrophotometer and the integrity of the RNA was confirmed through agarose gel electrophoresis.

RT-PCR

Synthesizing cDNA with AMV Reverse Transcriptase

In order to create *S. farnsworthianus* cDNA, first strand synthesis utilizing AMV Reverse Transcriptase (Promega, Madison, WI) was performed. In a 0.2 mL PCR tube, 10 µL of AMV RT 5X Buffer, 2 µL PCR nucleotides (10 mM) (Promega, Madison, WI), 1 µL RNAsin, 1 µL primer MTP1 3'C (50 pmol/µL), 1 µL AMV RT (10 U/µL), 1 µg DNAse treated total RNA, and nuclease free water were combined to make up a 50 µL total reaction volume. The reaction was incubated at 45°C for 45 minutes and then cooled to 20°C.

Traditional PCR

To produce the PCR product for cloning, traditional PCR was utilized for *S. farnsworthianus* cDNA. In a 0.2 µL PCR tube, 25 µL of 2X PCR Master Mix (Promega, Madison, WI), 1 µL MTP1 5'C (50 pmol/µL), 1 µL MTP1 3'C (50 pmol/µL), 5 µL of cDNA template, 17 µL nuclease free water, and 1 µL GoTaq Flexi polymerase (5 U/µL) (Promega, Madison, WI) were combined for a 50 µL reaction. The PCR cycling times were: 1 cycle of 95°C for 5 minutes, 30 cycles at 95°C denaturation for 1 minute, 52°C annealing for 2 minutes, and 72°C extension for 2 minutes, and after the last cycle the reaction was cooled down to 20°C. The PCR products were run on a 1% agarose gel to confirm that the target gene (*MTP1* at predicted 1,250bp) was amplified.

PCR Modification of *S. polygaloides MTP1*. SP1+2-7C, an *MTP1* gene from *S. polygaloides,* was acquired from the DNA stocks of Sharifa Llemit (Llemit Honors Thesis, 2005). After sequencing, it was revealed that the stop codon for this particular *MTP1* sequence was reversed from TTA to ATT. To correct this error, a PCR reaction utilizing Pfu Ultra polymerase (Invitrogen, Grand Island, NY) was performed. This polymerase was chosen

because of its high fidelity and proof reading ability. In a 0.2 µL PCR tube, 40.6 µL of nuclease free water, 5 µL 10X Pfu Ultra Buffer, 0.4 µL PCR nucleotides (10 mM) (Promega, Madison, WI), 1 µL DNA template, 1 µL MTP1 5'B (50 pmol/µL), 1 µL MTP1 3'B (50 pmol/µL), and 1 µL Pfu Ultra polymerase (2.5 U/µL) were combined to make up a 50 µL reaction. Four PCR reactions were run, each using 0.1 ng/µL, 1 ng/µL, 10 ng/µL, and 100 ng/µL of SP 1+2-7C DNA template. The cycling protocol was 1 cycle for 95°C, 30 cycles of 95°C denaturation for 30 seconds, 52°C annealing for 30 seconds, and 72°C extension for 1 minute. Then, 1 cycle at 72°C for final extension for 10 minutes and the product was cooled down to 20°C. After running 10 µL of each of the products on a 1% agarose gel, the PCR product created with 10 ng/µL of DNA template was chosen for cloning and bacterial transformation and thereafter known as SPY.

Table 1. Brassicaceae species primers used for RT-PCR. All Primers were ordered from IDT DNA Technologies (Coralville, IA).

Sequence Name	**Consensus Sequence (5' TO 3')**	**Tm (°C)**	**Restriction Enzyme Site**
MTP1 5'C	ATC TCG AGA TGG AGT CTT CAA GTC CCC ACC ATA GTC A	71.3	XhoI 5' C˅TCGAG 3'
MTP1 3'C	ATC TCG AGT TAG CGC TCG ATT TGT AT	63	XhoI 5' C˅TCGAG 3'
MTP1 5'B	GCA AGC TTA TGG AGT CTT CAA GTC CCC ACC ATA GTC A	68.3	HindIII 5' A˅AGCTT 3'
MTP1 3'B	ATG AAT TCT TAG CGC TCG ATT TGT AT	55.0	EcoRI 5' G˅AATTC 3'

Phenol/Chloroform Purification of DNA

Before cloning into the appropriate vector, RT-PCR products from *S. farnsworthianus* and *S. polygaloides* were cleaned using a phenol/chloroform extraction procedure. In a 1.5 mL microcentrifuge tube, 40 µL of cDNA was combined with 220 µL nuclease free water and 250 µL phenol/chloroform (25:24:1) and mixed well. The tube was spun for 10 minutes at 14,000 xg. Then, the supernatant was transferred to a new 1.5 mL microcentrifuge tube, and mixed with 125 µL of 7.5 M ammonium acetate and 1 mL of isopropanol. To better pellet the cDNA, the tube was chilled at -80°C for 30 minutes and then spun for 10 minutes at 14,000 xg. The supernatant was decanted and the pellet was washed with 500 µL of ethanol and spun for 5 minutes at 14,000 xg. After the excess liquid was pipetted off, the pellet was air dried for 15 minutes at room temperature and resuspended in 40 µL of nuclease free water. All purified cDNAs were quantified via a Nanodrop spectrophotometer and was stored at -20°C.

Cloning *MTP1* cDNA's into Vector *pYES2*

Cloning the MTP1 protein into *pYES2*

The purified *S. farnsworthianus MTP1* (*SfMTP1*) RT-PCR product was subcloned into the *E.* coli/*S. cerevisiae* shuttle vector *pYES2* (allowing for ampicillin selection) via a restriction enzyme digestion. In a 1.5 mL microcentrifuge tube, 3 µL of 10X buffer D, 1 µL pYES2 plasmid (100 ng/µL) (Invitrogen, Grand Island, NY), 10 µL purified DNA (100 ng/µL), 1 µL XhoI (10 U/µL) (Promega, Madison, WI), and 15 µL nuclease free water were combined and incubated at 37°C for 90 minutes. The 30 µL restriction enzyme digest was combined with 220 µL of sterile distilled water and underwent phenol/chloroform purification again to prevent any contaminants from interfering with ligation and transformation procedures. The resulting pellet,

was resuspended in 17 µL of sterile distilled water and immediately stored at -20°C for further use or used in the ligation procedure.

The sequence corrected *S. polygaloides MTP1* (*SpMTP1*) RT-PCR product was also inserted into vector *pYES2* via digestion. In a 1.5 mL microcentrifuge tube, 3 µL of 10X buffer E, 1 µL *pYES2* plasmid (100 ng/µL), 5 µL purified DNA, 1 µL HindIII (10 U/µL), 1 µL EcoRI (10 U/µL) (Promega, Madison, WI) and 19 µL nuclease free water were combined and incubated at 37°C for 4 hours. This digest was cleaned via phenol/chloroform purification and then resuspended in 17 µL for ligation.

Ligation

To ligate the *SfMTP1* insert with the *pYES2* vector, the entire 17 µL from the cleaned digest was combined with 2 µL of 10X Ligase buffer (Promega, Madison, WI) and 1 µL of T4 ligase (3 U/mL) (Promega, Madison, WI). This reaction was incubated at room temperature (22°C) for 16 to 18 hours. The *SpMTP1* insert was ligated at 30°C overnight.

Bacterial Transformation and *MTP1* Confirmation

Transformation of *MTP1* cDNA into Chemically Competent Topp10F$^-$ *E. coli*

After ligation of the *SfMTP1* or *SpMTP1* insert into the bacterial/yeast shuttle vector *pYES2*, the ligation products were transformed into Topp10F$^-$ *E. coli* chemically competent cells. Ten microliters of the ligation reaction was added to 100 µL of chemically competent cells. This mixture was incubated on ice for 35 minutes. The cells were heat shocked at 42°C for 90 seconds in a water bath. Immediately after heat shock, 1 mL of Luria Broth (LB) was added to the microcentrifuge tube and the tube was placed in the incubator shaker. Bacterial cells were incubated at 37°C and shaken at 220 rpm for 60 minutes. Following this recovery stage, the cells were plated with a cell spreader on petri dishes containing LB + AMP (200 µg/mL) media at

either concentrations of 100 µL or 900 µL of cells. The bacterial cells were grown overnight at 37°C.

Miniprep

Randomly selected bacterial colonies from petri dishes were miniprepped. A 1 mL starter culture was prepared for each potential colony and grown overnight at 37°C (shaken at 220 rpm) in LB + AMP (200 µg/mL). The next day, the bacterial cells were spun for 30 seconds to pellet the bacteria and then resuspended in 50-100 µL of LB + AMP. From a Qiagen kit (Qiagen, Valencia, California), 210 µL of G2 lysis buffer was mixed into the cells and then they were incubated at room temperature for 5 minutes. Then, 280 µL of G3/M3 neutralization buffer was added to the tube and it was spun for 10 minutes at 14,000 xg. The resulting supernatant was collected in a new 1.5 mL microcentrifuge tube. To pellet the DNA, 700 µL of isopropanol was mixed with the supernatant and then spun for 10 minutes at 14,000 xg. The pellet was washed with 500 µL of 70% ethanol and centrifuged for 5 minutes at 14,000 xg. After air drying for 15 minutes, the pellet was resuspended in 40-50 µL of sterile distilled water.

Restriction Enzyme Digest

From each miniprepped tube of cells, 20 µL of DNA was used for restriction enzyme digestion. All restriction enzymes and buffers were ordered from Promega (Madision, WI). To digest *SfMTP1* clones, 20 µL DNA, 3 µL 10X Buffer D, 1 µL RNAse A (10 mg/mL) (Promega, Madison, WI), 1 µL XhoI (10 U/µL), and 5 µL of sterile distilled water were combined in a 1.5 mL microcentrifuge tube. The reaction was incubated at 37°C for 2 hours. To digest *SpMTP1* clones, 20 µL DNA, 3 µL 10X Buffer E, 1 µL RNAse A (10 mg/mL), 1 µL HindIII (10 U/µL), 1 µL EcoRI (10 U/µL) and 4 µL of sterile distilled water were combined in a 1.5 mL microcentrifuge tube. The reaction was incubated at 37°C for 4 hours. The entire 30 µL digest

was run on a 1% agarose gel to confirm which colonies most likely contained the *MTP1* genes. Clones of SF50c and SPY11 were then prepared for sequencing.

Plasmid Purification of Clones for Sequencing

Potential bacterial clones containing *SfMTP1* or *SpMTP1* genes were purified following protocol in the QIAfilter plasmid purification midi kit (Valencia, CA). A 50 mL bacterial culture, containing the *S. farnsworthianus MTP1* or *S. polygaloides MTP1*, was grown overnight using a 1 mL starter culture. The 50 mL bacterial culture was equally divided into two 50 mL tubes and centrifuged at 6,000 xg for 15 minutes at 4°C. The resulting pellets were resuspended in lysis buffers as explained in the QIAfilter Midi bench protocol and the lysates were passed through a filtered syringe (QIAGEN, 2005). The plasmid DNA was bound to the QIAGEN anion-exchange column under low-salt and pH conditions. A medium-salt wash was used to remove RNA, proteins, and other impurities from the filtered lysates. The plasmid DNA was eluted using a high-salt buffer and then mixed with seven-tenths volume of isopropanol to desalt and precipitate the plasmid DNA. Then, the sample was centrifuged for 30 minutes at 15,000 xg at 4°C. The resulting pellets were washed with 70% ethanol, centrifuged for 5 minutes at 15,000 xg at 4°C, and then air dried for 15 minutes. The purified DNA was combined and resuspended in a total of 125 μL-250 μL of sterile water. All samples were stored at -20°C.

DNA Sequencing

After the cloning and purification process, the DNA was sent out for sequencing to the University of Chicago sequencing center (Chicago, IL). *SfMTP1* potential clones were sequenced using vector specific primers GAL1F and CYC1R and the species specific internal primer SFInt (Table 2). *SpMTP1* potential clones were sequenced using GAL1F and CYC1R

and the species specific internal primer SPYInt (Table 2). Sequences retrieved were compiled and blasted on NCBI (2013) to confirm presence of *MTP1* gene.

Table 2. Brassicaceae sequencing primers for shuttle vectors *pYES2* and *pKYXL71*. All Primers were ordered from IDT DNA Technologies (Coralville, IA).

Sequence Name	Consensus Sequence (5' TO 3')	Tm (°C)
GAL1F	AAT ATA CCT CTA TAC TTT AAC GTC	47.5
CYC1R	GCG TGA ATG TAA GCG TGA C	54.3
SFInt	TCC GTG ACT GCG GGC TTT GA	61.8
SPYInt	TCC GCA TAG AAG CAG TGT GTT CCT	60.3
pKYLX 5'	TGA TAT CTC CAC TGA CGT AAG	58.7
pKYLXR	GGA TTC TGG TGT GTG CGC AAT GA	60.2

Yeast Transformation and Confirmation

Yeast Transformation

Once the desired *MTP1* gene was confirmed to be present in *pYES2*, the QIAGEN midi-prepped DNA was used to transform the metal sensitive *Saccharomyces cerevisiae* mutant strain *Δpep5::trp1*. This strain was chosen because of its metal sensitivity (Woolford *et al*., 1990). To transform the yeast strain, 3 two inch streaks of yeast were freshly grown on YPD agar plate for 36 hours. After yeast matured, 20-50 µL of the yeast was transferred to a 1.5 mL microcentrifuge tube using an autoclaved toothpick and the cells were resuspended in 1 mL of sterile double distilled water by vortexing. The tube of yeast was centrifuged for 5 seconds at 14,000 xg. The supernatant was removed and the resulting pellet was resuspended in 1 mL of 100 mM lithium acetate by vortexing. The reaction was then incubated for 5 minutes at 30°C. After incubation, the yeast cells were re-centrifuged for 5 seconds at 14,000 xg and the supernatant was removed. Next, the pellet was combined with 240 µL 50% w/v PEG, 36 µL1 M lithium acetate, 25 µL boiled salmon sperm DNA, and 50 µL plasmid DNA (2.5 µg total) and vortexed for 1 minute to completely resuspend the pellet. The yeast cells were heat shocked for 42°C in a water bath for 20 minutes. After the heat treatment, the yeast cells were spun for 10 seconds at 14,000 xg and the supernatant was removed. The pellet was then resuspended in 150 µL of sterile double distilled water. The entire 150 µL of cells was plated on a petri dish containing YNB + SC-TRP-URA+2% Glucose + 1.5% Agar plates and grown until colonies appeared (about 6-7 days).

Yeast Miniprep

Yeast colonies from the yeast transformation were re-streaked, with a sterile pipette tip, onto new YNB + SC-TRP-URA+2% Glucose + 1.5% Agar plates to be used as reference plates.

SfMTP1 and *SpMTP1* cDNAs were retrieved from the yeast through a yeast miniprep. The yeast colonies were each grown in YNB + SC-TRP-URA+2% Glucose at 30°C, while shaking at 220 rpm, until they reached an optical density (A600) between 2 and 3. One milliliter of the yeast cells was centrifuged at 14,000 xg for 20 seconds. The cell pellets were resuspended in 50-100 µL of yeast media by vortexing. Then 300 µL of TENS-Y (10 mM Tris, 1mM EDTA, 0.2 M sodium hydroxide, and 3% SDS) was mixed into the tube of yeast cells and it was incubated at room temperature for 15 minutes with intermittent mixing every 3 minutes. Subsequently, 150 µL of 3 M sodium hydroxide (pH 5.2) was mixed into the tube and then 500 µL of phenol/chloroform (25:24:1) was added. After centrifuging for 10 minutes at 14,000 xg, the upper phase was removed to a sterile 1.5 mL tube, combined with 700 µL of isopropanol and spun for 5 minutes at 14,000 xg. The resulting pellet was resuspended in 500 µL sterile distilled water, 50 µL 3 M sodium acetate, and 700 µL of isopropanol were added and the tube spun for 5 minutes at 14,000 xg. After decanting the supernatant, the pellet was washed with 500 µL 70% ethanol and centrifuged for 2 minutes at 14,000 xg. Finally, the pellet was air dried for 15 minutes and resuspended in 40 µL of sterile distilled water.

PCR Confirmation Performed for EV1 and SF50c-8Y

In order to visually confirm, on a 1% agarose gel, that the *MTP1* gene was successfully transformed into the yeast, purified DNA was amplified through traditional PCR for yeast clones EV1 and SF50c-8Y. Three PCR reactions were set up for each clone using 1 µL, 5 µL, and 10 µL of DNA template. In the 0.2 µL PCR tubes, 25 µL of 2X PCR Master Mix (Promega, Madison, WI), 1 µL GAL1F (50 pmol/µL), 1 µL CYC1R (50 pmol/µL), DNA template, nuclease free water, and 1 µL GoTaq Flexi polymerase (5 U/µL) (Promega, Madison, WI) were combined for a 50 µL reaction. The PCR cycles were as follows: 95°C for 5 minutes, 30 cycles of 95°C

denaturation for 1 minute, 52°C annealing for 2 minutes, and 72°C extension for 2 minutes, and final cooldown to 20°C. The entire PCR product was run on a 1% agarose gel to confirm that the target gene (*MTP1* at about 1,250bp) was amplified.

Bacterial Confirmation Performed for SPY11-7Y

The *SpMTP1* from the yeast was inserted into the bacterial/yeast shuttle vector *pYES2*. From the purified DNA, 10 µL was used to transform Topp10F^{-} chemically competent *E. coli* cells. Following the recovery stage, the cells were plated on petri dishes containing LB + AMP (200 µg/mL ampicillin concentration) media at concentrations of 100 µL and 900 µL. The bacterial cells were allowed to grow overnight at 37°C. Seven colonies appeared on the plates and these were referred to as SPY-1Y to SPY-7Y. These bacterial colonies were minipreped and amplified through PCR using the following recipe and conditions. In a 0.2 µL PCR tube, 25 µL of 2X PCR Master Mix (Promega, Madison, WI), 1 µL MTP1 5'B (50 pmol/µL), 1 µL MTP1 3'B (50 pmol/µL), 5 µL of cDNA template, 17 µL nuclease free water, and 1 µL GoTaq Flexi polymerase (5 U/µL) (Promega, Madison, WI) were combined for a 50 µL reaction. The PCR reaction time included: 1 cycle of 95°C for 5 minutes, 30 cycles of 95°C denaturation for 1 minute, 52°C annealing for 2 minutes, and 72°C extension for 2 minutes, and lastly cooled down to 20°C. After running the whole reaction on a 1% agarose gel, SPY11-7Y was confirmed to have the *MTP1* gene.

Yeast Metal Tolerance Assay

Metal Tolerance Assay

Yeast confirmed to have the appropriate *MTP1* plasmids were assayed for metal tolerance. Fifteen streaks of yeast (5 cm long and half a centimeter wide) were grown for 6 days at 30°C on YNB+A+SC-TRP-URA+2% Glucose +1.5% Agar. On the sixth day, freshly

autoclaved YNB+A+SC-TRP-URA+4% Galactose +1.5% Agar was poured into fifteen 50 mL tubes and equilibrated in a waterbath at 48°C for 30-60 minutes to keep the agar from solidifying. The 15 yeast streaks were separated into individual 1.5 mL microcentrifuge tubes and the yeast was suspended in 1 mL of YNB+A+SC-TRP-URA+4% Galactose by vortexing until the cells were completely resuspended. One hundred microliters of this yeast was resuspended in 900 µL of YNB+A+SC-TRP-URA+4% Galactose. The optical density of the yeast was measured at A600 nm. The following formula was used to calculate the total volume of cells to put in the autoclaved media for the metal tolerance assay.

$$\frac{(10^6 \text{ cells})\ 1000}{(\text{Absorbance at 600 nm})\ 10^6} = \text{X } \mu\text{L}$$

The calculated volume of cells was taken from the original suspension of the yeast and was pipetted into the 50 mL media in the 48°C water bath. After inverting the tube several times, the yeast cells and media were slowly poured into a 150 mm x 15 mm petri dish. After the media solidified, sterile tweezers were used to place four sterile 0.75 cm diameter paper cloning disks on top of the media of each individual dish. All of the disks on the media were evenly distributed on the surface of the plate. A metal solution was then pipetted onto the center of the disk. Metals and corresponding volumes used were: 20 µL of 400 mM nickel acetate, 20 µL of 400 mM Zinc acetate, 5 µL of 400 mM Cobalt acetate, and 2 µL of 4 mM Cadmium acetate. One metal was used per petri dish. This procedure was replicated with all metals between five and seven times. When the disks completely absorbed the metal solution, the plates were transferred to an incubator and grown for 6 days at 30°C. On the sixth day, circular areas lacking yeast growth could be distinguished around the cloning disks and represented the zone of inhibition. The diameter of the circular area was measured in millimeters using a metric ruler and then the area of the circle was calculated.

Statistics

The average area of the zones of inhibition were taken for each petri dish and analyzed via SPSS (version 21.0). Petri dishes with high cell density were chosen for investigation. Plates with low cell densities were omitted from statistical calculations. A Kruskal-Wallis analysis was used to compare the areas between EV1, SF50c-8Y, and SPY11-7Y and determine if at least one of the genes was different from the other genes. The data was then applied to Tukey's HSD (honestly significant difference) test to identify genes that were significantly different in their metal tolerance.

Insertion of *MTP1* into Vector *pKYLX71*

Digest *MTP1* Qiagen Prep DNA into *pKYLX71*

The *SfMTP1* Qiagen Prep was subcloned into the *Agrobacterium* tumefaciens/Plant transformation vector *pKYLX71* via a restriction enzyme digestion. In a 1.5 mL microcentrifuge tube, 3 µL of 10X buffer D, 1 µL *pKYLX71* plasmid (100 ng/µL), 8µL purified DNA (137 ng/µL), and 1 µL XhoI (10 U/µL) were combined and incubated at 37°C for 2 hours. The 30 µL digest was combined with 220 µL of sterile distilled water and was cleaned via the phenol/chloroform purification procedure. The resulting pellet, from the purification, was resuspended in 17 µL of sterile distilled water and immediately stored at -20°C for further use.

The *SpMTP1* Qiagen Prep was also inserted into the vector *pKYLX71* via digestion. In a 1.5 mL microcentrifuge tube, 3 µL of 10X buffer E, 1 µL *pKYLX71* plasmid (100 ng/µL), 1µL purified DNA (100 ng/µL), 1 µL HindIII (10 U/µL), 1 µL XbaI (10 U/µL), and 23 µL of sterile distilled water were combined and incubated at 37°C for 3 hours. The 30 µL digest was combined with 220 µL of sterile distilled water and was cleaned via the phenol/chloroform

purification procedure. The resulting pellet, from the purification, was resuspended in 17 µL of sterile distilled water and immediately stored at -20°C for further use.

Ligation, Bacterial Transformation, and Miniprep

To ligate the *MTP1* insert with the *pKYLX71* plant shuttle vector, the entire 17 µL from the cleaned digest was combined with 2 µL of 10X Ligase buffer (Promega, Madison, WI) and 1 µL of T4 ligase (3 U/µL). For both *SfMTP1* and *SpMTP1* inserts, the reaction was incubated at 4°C for 16 to 18 hours. The ligated reaction was then transformed into Topp10F^{-} chemically competent *E. coli* cells via the chemically competent cell transformation procedure. Potential clones were randomly selected from LB + TET (25 µg/mL tetracycline) selection plates and miniprepped.

Restriction Digest, Plasmid Purification, and Sequencing

To digest *SfMTP1* clones into *pKYLX71*, 20 µL DNA, 3 µL 10X Buffer D, 1 µL RNAse A (10 mg/mL) (Promega, Madison, WI), 1 µL XhoI (10 U/µL), and 5 µL of sterile distilled water were combined in a 1.5 mL microcentrifuge tube. The reaction was incubated at 37°C for 2 hours. To digest *SpMTP1* clones, 20 µL DNA, 3 µL 10X Buffer E, 1 µL RNAse A (10 mg/mL), 1 µL HindIII (10 U/µL), 1 µL XhoI (10 U/µL), and 4 µL of sterile distilled water were combined in a 1.5 mL microcentrifuge tube. This reaction was incubated at 37°C for 4 hours. The entire digest was run on a 1% agarose gel to confirm which colonies most likely contained the *MTP1* gene in the *pKYLX71* vector. SF50c-118 and SPY11-32 was prepared for sequencing.

After the cloning and purification process, the DNA was sent out for sequencing to University of Chicago sequencing center (Chicago, IL). *SfMTP1* potential clones were sequenced using vector specific primers pKYLX 5' and pKYLXR and the species specific internal primer SFInt (Table 2). *SpMTP1* potential clones were sequenced using pKYLX 5' and pKYLXR and

the species specific internal primer SPYInt (Table 2). Sequences retrieved were compiled and blasted on NCBI (2013) to confirm presence of *MTP1* gene.

Agrobacterium Transformation

Once the sequencing results confirmed the presence of *MTP1* within vector *pKYXL71*, *Agrobacterium tumefaciens* was transformed with the *MTP1* clone. The *Agrobacterium* was electroporated with 100 ng total Qiagen miniprep purifiedDNA. After electroporation, the cells were resuspended in 1 mL of YPD and placed in a new 1.5 mL microcentrifuge tube. The cells were recovered by shaking them at 220 rpm, for 1 hour at 30°C. Next, the transformant were plated using *E.coli* plating procedures on YPD+Gen+Rif+Kan (YPD medium with 50 μg/mL rifampicin, 25 μg/mL gentamycin, and 50 μg/mL kanamycin) selection plates. The petri dishes were incubated at 30°C for 48 hours. Colonies that survived the screening from the three antibiotics were saved as glycerol stocks and one was picked at random for plant transfection.

Plant Transfection and Transgenic Plant Maintenance

Floral Dipping

One liter of *Agrobacterium* and the plasmid construct was grown at 28°C in YPD+Gen+Rif+Kan (100 μG/mL gentamycin, 50 μG/mL rifampicin, 30 μG/mL kanamycin) media until the optimal density (A600 nm) was between 1.5 and 2. The large culture was split into 4 sterile 250 mL centrifuge bottles and spun down at 500 xg at room temperature using a swinging bucket rotor. Next, the pellets were resuspended in 250 mL of infiltration media and combined in a 1 L beaker. Infiltration media was made of 2.2 g MS Salts + Gamborg's B5 vitamins, 50g sucrose, 0.5g MES (pH 5.7 with KOH), 0.044 μM benzylaminopurine, and 200 μL Silwet L-77. Once finished, the infiltration media was taken to the plant storage room to proceed with floral dipping. Tubs containing *A. thaliana* plants that had just flowered were previously set

aside for this procedure. The entire plant was immersed in the *Agrobacterium* solution. Once all the plants in a tub had been dipped, they were immediately wrapped with saran wrap. The following day, the plants were set upright and grown under a 9 hour light cycle (125 µEinsteins light intensity) at 23°C and 60% RH.

Seed Collection, Selection, and Growth

Seeds harvested from the transgenic *A. thaliana,* containing either *SfMTP1* or *SpMTP1*, were collected in separate zip lock bags and air dried. Once the plant material was dried, the seeds were collected using a small strainer to filter out the plant debris from the seeds. All seeds collected after these processes were stored in 1.5 mL microcentrifuge tubes with a large desiccation rock at room temperature. The next generation of transgenic plants was grown from these seeds. The transgenic *A. thaliana* seeds were surface sterilized according to the previous non-accumulator procedure; however, the seeds were instead plated on ½ X MS Media + Kanamycin (50 µg/mL concentration) + 1% agar petri dishes. The kanamycin allowed for selection of plants transformed with *Agrobacterium* containing the *MTP1* gene. All seedlings that survived the selection process were transplanted into hydroponics and the same selection procedures were replicated for future seeds until the T_3 generation.

CHAPTER III

RESULTS

SfMTP1 and *SpMTP1* Expression

RT-PCR amplifications run on an agarose gel confirmed presence of *S. farnsworthianus* and *S. polygaloides MTP1* gene predicted at approximately 1,250 bp (Figure 1). Both plant species were grown for about 2-3 months and then treated with 100 μM Ni for either 6 days or 12 days (Yessica Cerino unpublished methods). RT-PCR Products from both Ni treated and untreated *S. farnsworthianus* and *S. polygaloides* plants showed bands at approximately 1,250 bp for 6 day and 12 day samples.

Sequencing Analysis

MTP1 Nucleotide BLAST Analysis

Table 3 and Table 4 display the top BLASTn hits for *SfMTP1* and *SpMTP1* nucleotide sequences. The nucleotide BLASTn hits for *SfMTP1* and *SpMTP1* were very similar to *MTP1* coding sequences from Brassicaceae family members.

The top BLASTn hit for *SfMTP1* gene had 89% maximum identity with *Arabidopsis lyrata* metal transporter *MTP1* mRNA, complete coding sequence with an Error value of 0.0. Other high ranking BLASTn hits for the *SfMTP*1 nucleotide sequence included other variations of *A. lyrata* and *A. thaliana MTP* sequences with 88% or 89% maximum identities and Error values of 0.0. The top BLASTn hit for the *SpMTP1* gene had 89% maximum identity with the

Thlaspi arvense heavy metal transporter *MTP1* mRNA, complete coding sequence and had an Error value of 0.0.

Additional BLASTn hits for the *SpMTP1* nucleotide sequence were *A. lyrata* heavy metal transporter *MTP1* mRNA, complete coding sequence with an 89% maximum identity (Error value of 0.0) and various *A. thaliana* sequences with 88% maximum identities (Error values of 0.0).

MTP1 Protein BLAST Analysis

BLASTp results for SfMTP1 and SpMTP1 protein sequences identified putative conserved domain sequences (Figure 2). Both MTP1 sequences featured the cation efflux family domains (or cation diffusion facilitator family) which are associated with metal transport in plants and yeast (Paulsen and Saier, 1997; Delhaize *et al.*, 2003).

The top five protein BLASTp hits for SfMTP1 and SpMTP1 are shown in Table 5 and Table 6 respectively. The top BLASTp hit for both SfMTP1 and SpMTP1 was the zinc transporter ZAT from *A. thaliana* with an 86% maximum identity (Error value of 0.0) for SfMTP1 and an 85% maximum identity (Error value of 0.0) for SpMTP1. Other protein BLASTp hits for the two genes included non-accumulator and hyperaccumulator Brassicaceae family member MTPs and a *Brassica juncea* cation-efflux transporter with maximum identities ranging from 84% to 87% and Error values of 0.0.

SfMTP1and SpMTP1 sequences were aligned to determine differences between the two protein sequences in Figure 3 using the NCBI BLASTp suite (2013). The two genes were found to be 92% identical. In comparing the two sequences, it revealed an 8 amino acid gap at position 215 in SpMTP1 that is not present in SfMTP1.

Yeast Metal Tolerance Assays

Figure 4 shows examples of the yeast metal tolerance assay for metal sensitive yeast containing the empty vector *pYES2*, *SfMTP1*, and *SpMTP1* when exposed to Ni and Cd. Pictures displayed in Figure 4 were from assays showing significant differences in yeast metal tolerance by comparing their zones of inhibition.

Table 7 displays the calculated averages and standard deviations collected from the petri dishes of the yeast metal tolerance assays. When comparing the average areas of the zones of inhibition in the Ni metal tolerance assay, *SfMTP1* (SF50c-8Y) exhibited a 2.13-fold higher Ni tolerance than empty vector *pYES2* (EV1) and a 1.63-fold higher Ni tolerance than *SpMTP1* (SPY11-7Y). In the Zn metal tolerance assay, the *SfMTP1* and *SpMTP1* showed a mean 1.26-fold lower Zn tolerance than *pYES2*. In the Co metal tolerance assay, *pYES2*, *SfMTP1*, and *SpMTP1* all have similar Co tolerances. In the Cd metal tolerance assay, *SpMTP1* confers a 3.04-fold higher Cd tolerance than *pYES2* and a 1.77-fold higher Cd tolerance than *SfMTP1*.

Figure 5 displays a comparison of the average areas (mm^2) for the zones of inhibition for *pYES2*, *SfMTP1*, and *SpMTP1* when exposed to Ni, Zn, Co, and Cd. The threshold value for significance for all the tests was $p \leq 0.05$. The Kruskal-Wallis values for the Ni assay were $X^2=14.037$, 2 degrees of freedom (df), and $p=0.001$. This shows that one or more of the genes was significantly different from one or more of the other genes. Tukey's HSD test, with $p=0.000$, determined that all of the genes compared in the Ni metal tolerance assay were significantly different in their ability to impart increased resistance to the yeast. In Figure 5, the Ni metal tolerance assay clearly shows that the three genes give different metal tolerances to the yeast with *SfMTP1* exhibiting the highest Ni tolerance, then *SpMTP1*an intermediate tolerance, and lastly *pYES2* had the lowest Ni tolerance.

The Kruskal-Wallis values for the Zn assay were X^2=5.901, 2 df, and p=0.052. This showed that one or more of the genes were not significantly different from one or more of the other genes. Tukey's HSD gave a p=0.065 which showed that the metal tolerance imparted to the yeast between the genes has no significant difference. In Figure 5, the Zn metal tolerance assay shows that the *SfMTP1* and *SpMTP1* genes may confer lower Zn tolerance than *pYES2* due to the averages being slightly different than the control.

The Kruskal-Wallis values for the Co assay were X^2=4.522, 2 df, and p=0.104 showing that none of the genes' metal tolerance imparted to the yeast were significantly different from the other genes'. Tukey's HSD gave a p=0.078 which confirmed that there was no significant difference in metal tolerance imparted to the yeast. Figure 5 also shows that *pYES2, SfMTP1*, and *SpMTP1* all have a similar tolerance to Co.

The Kruskal-Wallis values for the Cd assay were X^2=10.681, 2 df, and p=0.005 which showed that one or more of the genes was different from the other genes. Tukey's HSD, with p=0.000, verified that all of the genes were significantly different from the other genes. Figure 5 shows that *SpMTP1* infers the highest Cd tolerance from the three genes, second is *SfMTP1*, and then *pYES2*.

State of Transgenic Plants

Currently, *SpMTP1* transgenic *A. thaliana* plants are being selected and grown until the T_3 generation is produced for future metal tolerance investigation. *SfMTP1* has been transformed into the *Agrobacterium* and awaits plant transformation. The *MTP1* genes and their insertion into the *A. thaliana* genome seems to have caused no ill effects on the transgenic plants; indicated by no change in plant morphology and their ability to produce viable seeds.

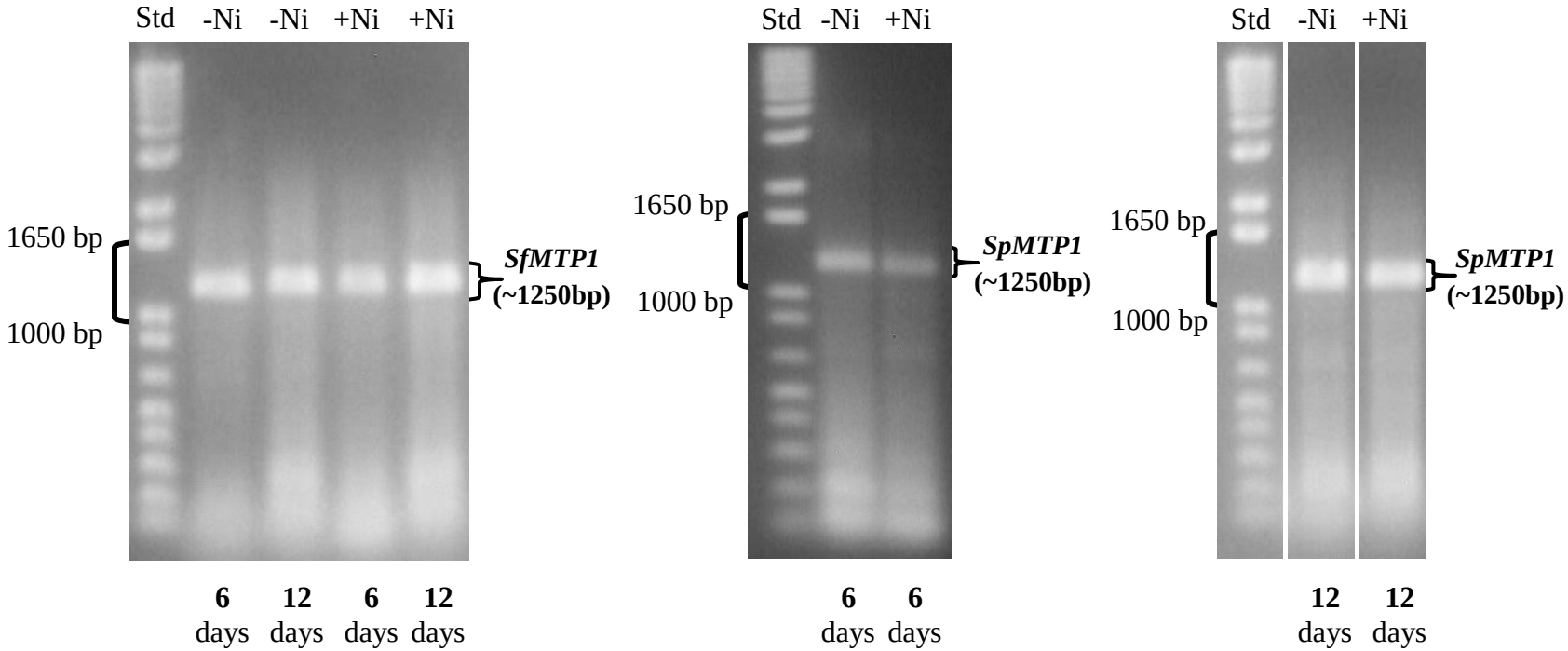


Figure 1. Nonquantitative RT-PCR amplification using *MTP1* primers for *S. farnsworthianus* and *S. polygaloides* 6 days and 12 days control and Ni treated. After accumulator plant species matured in the plant hydroponics tub, several tubs were separated and treated with 100 μM Ni for either 6 days or 12 days. Untreated and Ni treated plants were grown under the same conditions. RT-PCR results for Ni treated *S. farnsworthianus* and *S. polygaloides* were contributed by Yessica Cerino (unpublished results). Nonquantitative RT-PCR indicates that *SfMTP1* and *SpMTP1* is expressed in both Ni treated and untreated plants.

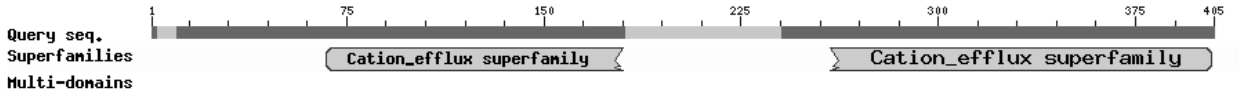
Table 3. Top BLASTn hits for *S. farnsworthianus MTP1*

Description	**Max Identity**	**E value**	**Accession**
A. lyrata heavy metal transporter *MTP1* mRNA, complete cds	89%	0.0	AY483147.1
Arabidopsis lyrata subsp. lyrata heavy metal transporter *MTP2*, mRNA	88%	0.0	XM_002880219.1
Arabidopsis lyrata subsp. petraea partial mRNA for putative zinc transport protein MTP1-1	89%	0.0	AJ704807.1
Arabidopsis thaliana chromosome 2, complete sequence	88%	0.0	CP002685.1
Arabidopsis thaliana AT2G46800 mRNA, complete cds, clone: RAFL22-29-C05	88%	0.0	AK317164.1

Table 4. Top BLASTn hits for *S. polygaloides MTP1*

Description	**Max Identity**	**E value**	**Accession**
Thlaspi arvense heavy metal transporter *MTP1* mRNA, complete cds	89%	0.0	AY483145.1
Arabidopsis lyrata heavy metal transporter *MTP1* mRNA, complete cds	89%	0.0	AY483147.1
A. thaliana chromosome 2, complete sequence	88%	0.0	CP002685.1
A. thaliana AT2G46800 mRNA, complete cds, clone: RAFL22-29-C05	88%	0.0	AK317164.1
A. thaliana mRNA for putative zinc transporter, complete cds, clone: RAFL06-72-J05	88%	0.0	AK226483.1

SfMTP1 protein sequence conserved domains



- **SpMTP1 protein sequence conserved domains**

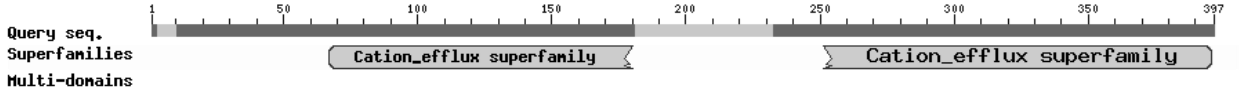


Figure 2. Protein sequence putative conserved domains in SfMTP1 and SpMTP1. The protein domains include the characteristic cation efflux family domains associated with metal transport in plants and yeast.

Table 5. Top BLASTp hits for *S. farnsworthianus* MTP1

Description	**Max Identity**	**E value**	**Accession**
zinc transporter ZAT [*A.thaliana*]	85%	0.0	AAD11757.1
heavy metal transporter MTP2 [*A. lyrata* subsp. *lyrata*]	87%	0.0	XP_002880265.1
metal tolerance protein [*A. halleri* subsp. *halleri*]	84%	0.0	CAY39366.1
hypothetical protein CARUB_v10023370mg [*Capsella rubella*]	87%	0.0	EOA27251.1
cation-efflux transporter [*Brassica juncea*]	87%	0.0	AAO83659.1

Table 6. Top BLASTp hits for *S. polygaloides* MTP1

Description	**Max Identity**	**E value**	**Accession**
zinc transporter ZAT [*A. thaliana]*	86%	0.0	AAD11757.1
metal tolerance protein [*Arabidopsis halleri* subsp. *halleri*]	84%	0.0	CAY39366.1
heavy metal transporter MTP2 [*A. lyrata* subsp. *lyrata*]	86%	0.0	XP_002880265.1
heavy metal transporter MTP2 [*A. lyrata*]	84%	0.0	AAR83909.1
metal tolerance protein 1 short form [*A. thaliana*]	86%	0.0	NP_182203.1

SpMTP1 and SfMTP1 protein alignment:

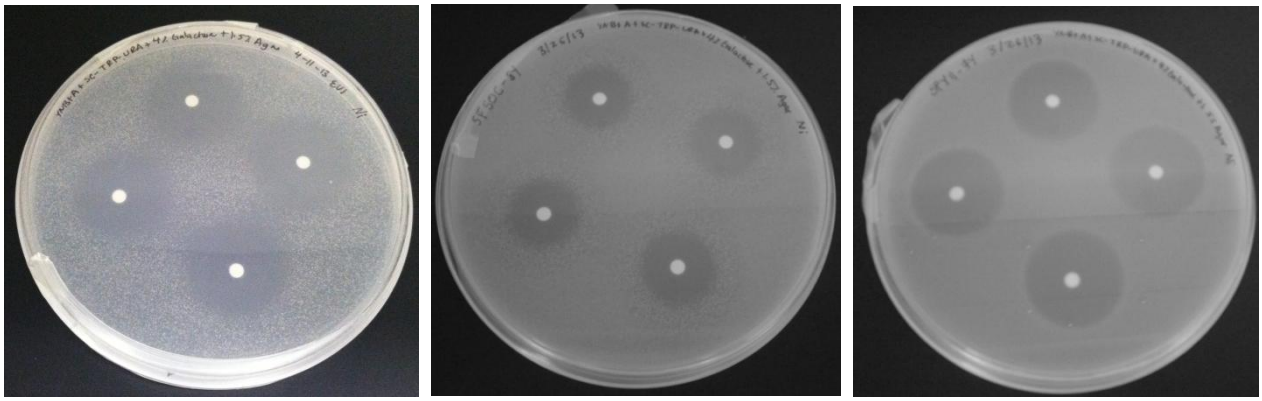
Identities: 373/405 (92%)

```
SpMTP1 1    MESSSPHHSHIIEVNVGKSDEEQTAGASKVCGEAPCEFSDLKNASGDAEEHTASMRKLCI  60
            MESSSPHHSHIIEVNV KSDEE+T+G SK CGEAPC FSDLKNA GDAEE  ASMRKLCI
SfMTP1 1    MESSSPHHSHIIEVNVRKSDEERTSGGSKACGEAPCGFSDLKNAPGDAEERNASMRKLCI  60

SpMTP1 61   AVALCLLFMTVEVFGGIKANSLAILTDAAHLLSDVAAFAISLFSLWAAGWEATPRQTYGF  120
            AV LCLLFMTVEVFGGIKANSLAILTDAAHLLSDVAAFAISLFSLWAAGWEATPRQTYGF
SfMTP1 61   AVVLCLLFMTVEVFGGIKANSLAILTDAAHLLSDVAAFAISLFSLWAAGWEATPRQTYGF  120

SpMTP1 121  FRIEILSALVSIQLIWLLTGILVYEAIIRLLTETSEVNGFLMFLVAAFGLVVNIIMAVLL  180
            FRIEIL ALVSIQLIWLLTGILVYEAI RLLTETSEVNGFLMFLVAAFGLVVNIIMAVLL
SfMTP1 121  FRIEILGALVSIQLIWLLTGILVYEAITRLLTETSEVNGFLMFLVAAFGLVVNIIMAVLL  180

SpMTP1 181  GHDHGHSHGHGHSHGHDHHNHGGNHSHGVTVTTHH--------GHGHGHNHGEDKHHAHG  232
            GHDHGHSHGHGH HGHDHHNHGGNHSHGVTVTTHH         H HGH+HGEDKHHAHG
SfMTP1 181  GHDHGHSHGHGHGHGHDHHNHGGNHSHGVTVTTHHHHDHDHDHDHDHGHSHGEDKHHAHG  240

SpMTP1 233  DVTEQLLDKSKPPVADKEKRKRNINVQGAYLHVLGDSIQSVGVMIGGAIIWYKPEWKIVD  292
            DVTEQLLDKSKP V DKEKRKRNINVQGAYLHVLGDSIQSVGVMIGGAIIWY P+WKIVD
SfMTP1 241  DVTEQLLDKSKPAVTDKEKRKRNINVQGAYLHVLGDSIQSVGVMIGGAIIWYNPKWKIVD  300

SpMTP1 293  LICTLVFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAI  352
            L CTLVFSVIVLGTTINMIR+ILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAI
SfMTP1 301  LTCTLVFSVIVLGTTINMIRSILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIWAI  360

SpMTP1 353  TVGKVLLACHVNISPEADADMLLNKVIDYIRREYNISHVTIQIER  397
            TVGKVLLACHVNI PEADADM+LNKVIDYIRREYNISHVTIQIER
SfMTP1 361  TVGKVLLACHVNIRPEADADMVLNKVIDYIRREYNISHVTIQIER  405
```

Figure 3. Sequence alignment of SpMTP1 and SfMTP1 showing 92% identity. A large gap at site 215 may account for the different metal tolerance characteristics between the two plant species. Gaps in the alignment represent non-similar amino acids. The "+" indicates similar amino acids and letters indicate the same amino acid. Protein sequences were aligned using the NCBI BLASTp suite (2013).

Ni Treated Samples

Empty Vector 1 SF50c-8Y SPY11-7Y



Cd Treated Samples

Empty Vector 1 SF50c-8Y SPY11-7Y

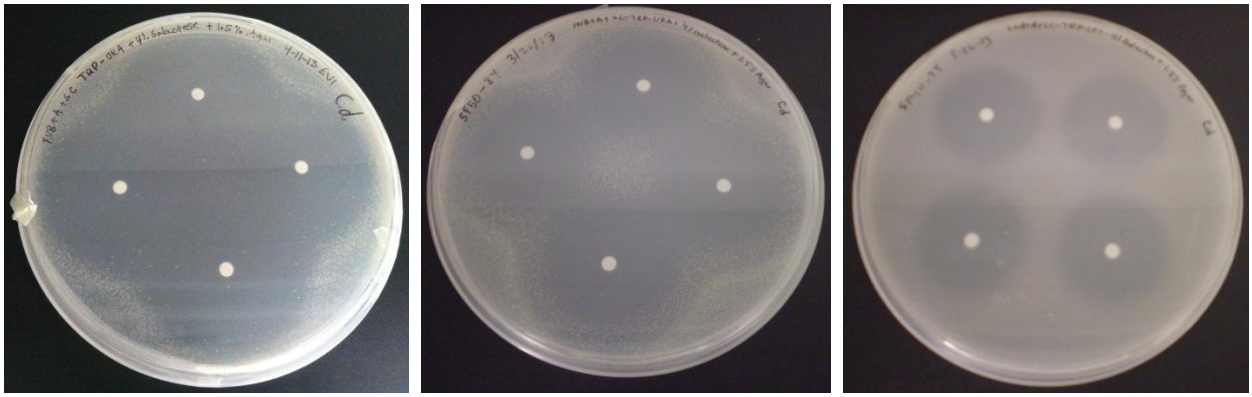


Figure 4. Representative examples of yeast metal tolerance assay plates displaying the zones of inhibition of *Δpep5::trp1* yeast containing the empty vector *pYES2* (Empty Vector 1, left), *Δpep5::trp1* yeast containing *pYES2* with *SfMTP1* (SF50c-8Y, middle), and *Δpep5::trp1* yeast containing *pYES2* with *SpMTP1* (SPY11-7Y, right).

Table 7. Zone of inhibition averages and standard deviations per petri dish calculated from yeast metal tolerance assays for*Δpep5::trp1* yeast containing the empty vector *pYES2* (EV1), *Δpep5::trp1* yeast containing *pYES2* with *SfMTP1* (SF50c-8Y), and *Δpep5::trp1* yeast containing *pYES2* with *SpMTP1* (SPY11-7Y). These values were used for statistical analysis. Sample size "n" represents number of petri dish assayed.

	Average Area ±Standard Deviations for Zone of Inhibition (mm^2)			
	400 mM Ni	**400 mM Zn**	**400 mM Co**	**4 mM Cd**
pYES2	837.92±63.12 (n=6)	587.18±61.83 (n=6)	903.60±60.32 (n=5)	2305.34±177.12 (n=4)
SfMTP1	393.83±57.14 (n=4)	751.09±130.19 (n=4)	1029.00±95.09 (n=3)	1339.20±109.92 (n=4)
SpMTP1	642.90±52.56 (n=7)	728.91±72.46 (n=3)	902.62±61.45 (n=4)	757.44±75.19 (n=5)

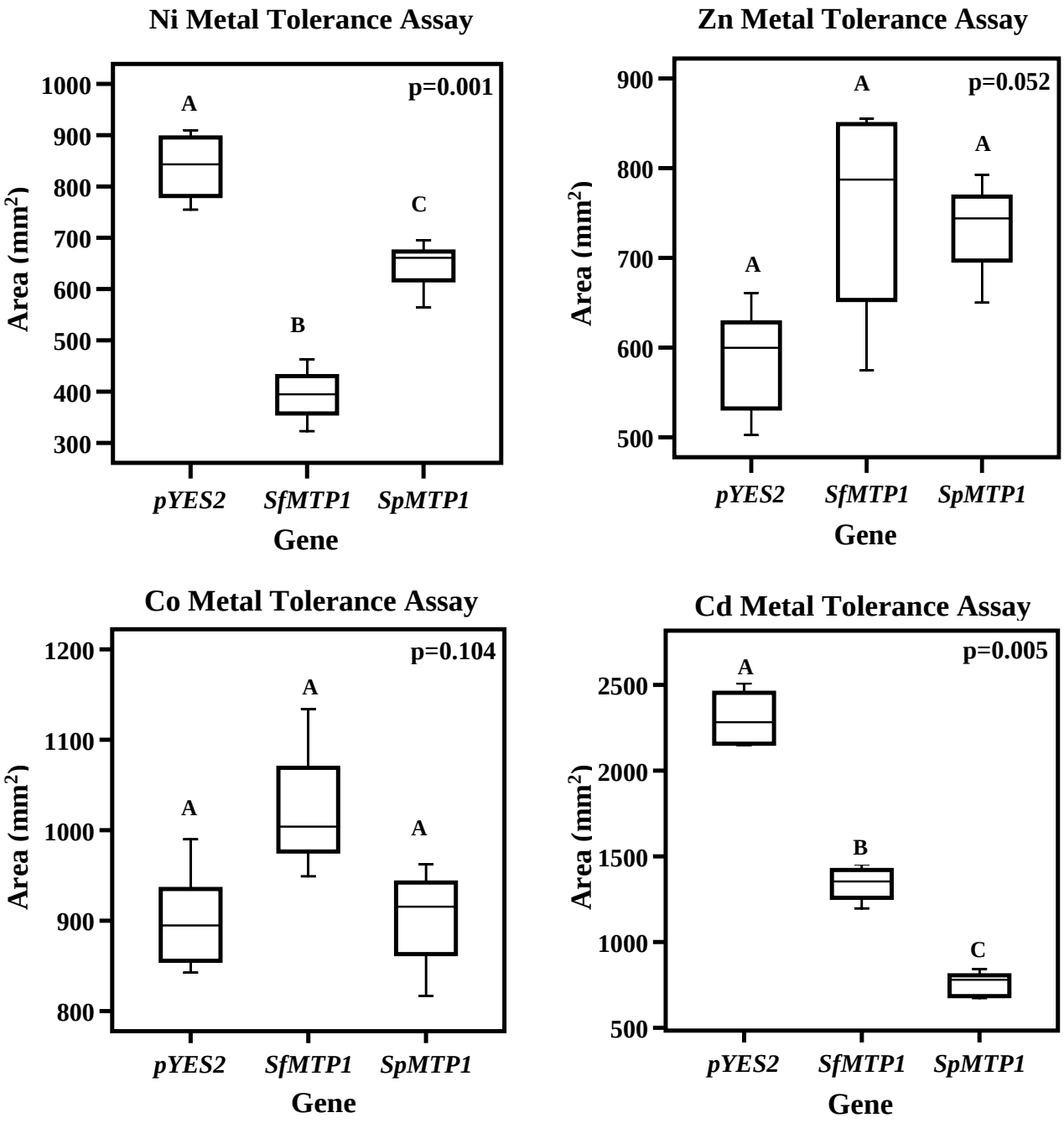


Figure 5. The average zones of inhibition (mm^2) for yeast containing genes *pYES2*, *SfMTP1* and *SpMTP1*. Box and whiskers represent zones of inhibition for yeast metal tolerance assays (400 mM Ni, 400 mM Zn, 400 mM Co, and 4 mM Cd). Genes showing different letters above the standard deviation line differ significantly ($p \leq 0.05$) from the other groups of genes. Genes showing same letters above the standard deviation line do not differ significantly from the each of the groups.

CHAPTER IV

DISCUSSION

Gene Expression

Nonquantitative RT-PCR shows that both *SfMTP1* and *SpMTP1* are expressed in Ni treated and untreated plant shoots at 6 days and 12 days after metal treatment. These results indicate that the accumulator and hyperaccumulator genes are constitutively expressed in the leaves of these plant species. Therefore, regardless of Ni exposure, *S. farnsworthianus* and *S. polygaloides MTP1* gene expression do not appear to be affected by exposure to Ni. Knowledge of the constitutive expression of *SfMTP1* in *S. farnsworthianus* and *SpMTP1* in *S. polygaloides* are crucial for understanding how *MTP1* expression affects plant metal tolerance and uptake. To make conclusions about the *MTP1* mechanism, it is necessary to know at what times and in which location the *MTP1* genes are being expressed. The nonquantitative RT-PCR shows that the *MTP1* genes in both plants are being expressed at all times regardless of Ni treatment; therefore, the level of *MTP1* gene expression may not account for the different metal accumulation characteristics observed among *Streptanthus* plant species. Another factor such as the lack of protein expression or protein sequence may be the cause of differences in Ni accumulation between the species.

Sequencing Analysis

Both *SfMTP1* and *SpMTP1* nucleotide sequences shared a high level of homology with *MTP1* gene sequences from other members of the Brassicaceae family. In addition, SfMTP1 and

SpMTP1 protein sequences shared a high level of identity with the *A. thaliana* ZAT/AtMTP1 protein sequence. The top BLAST hits for both nucleotide and protein sequences give strong evidence that the cloned genes are MTP1 gene family members.

Furthermore, putative cation efflux conserved domains revealed that SfMTP1 and SpMTP1 proteins possess the cation diffusion facilitator family signature that is used to identify members of the CDF family (Paulsen and Saier, 1997). This is important because all CDF family members have the specific ability to transport heavy metal ions (Paulsen and Saier, 1997). In analyzing the conserved domain, Figure 2 reveals that both SfMTP1 and SpMTP1 possess all the sequence features consistent with known CDF family members. These attributes include a CDF signature sequence, six transmembrane domains, and a variable histidine-rich sequence located in the middle of the protein specifically located after the fourth and fifth loops of the transmembrane domain (Paulsen and Saier, 1997; Persans *et al*., 2001). A gap in the middle of the cation efflux domain in Figure 2 represents the variable histidine-rich region that is common to all CDF family members. When comparing SfMTP1 and SpMTP1 proteins to other hyperaccumulator and non-accumulator protein sequences of Persans *et al.* (2001), a gap is easily identified in the second variable histidine rich region (Figure 6). In addition, $(HX)_n$ duplications discussed by Paulsen and Saier (1997) can be identified within the histidine regions which are believed to function in metal ion binding.

Protein sequence alignments of SfMTP1 and SpMTP1 revealed that the two sequences were 92% identical. The sequences differed by 32 amino acids. However, the 8 amino acid gap at position 215 in SpMTP1 was prominent (Figure 2 and Figure 6). This is significant, because it occurs in the second histidine region of the sequence. The results suggest that this gap may

affect MTP1 protein regulation of metal transport in *S. polygaloides* and contributing to its known status as a hyperaccumulator.

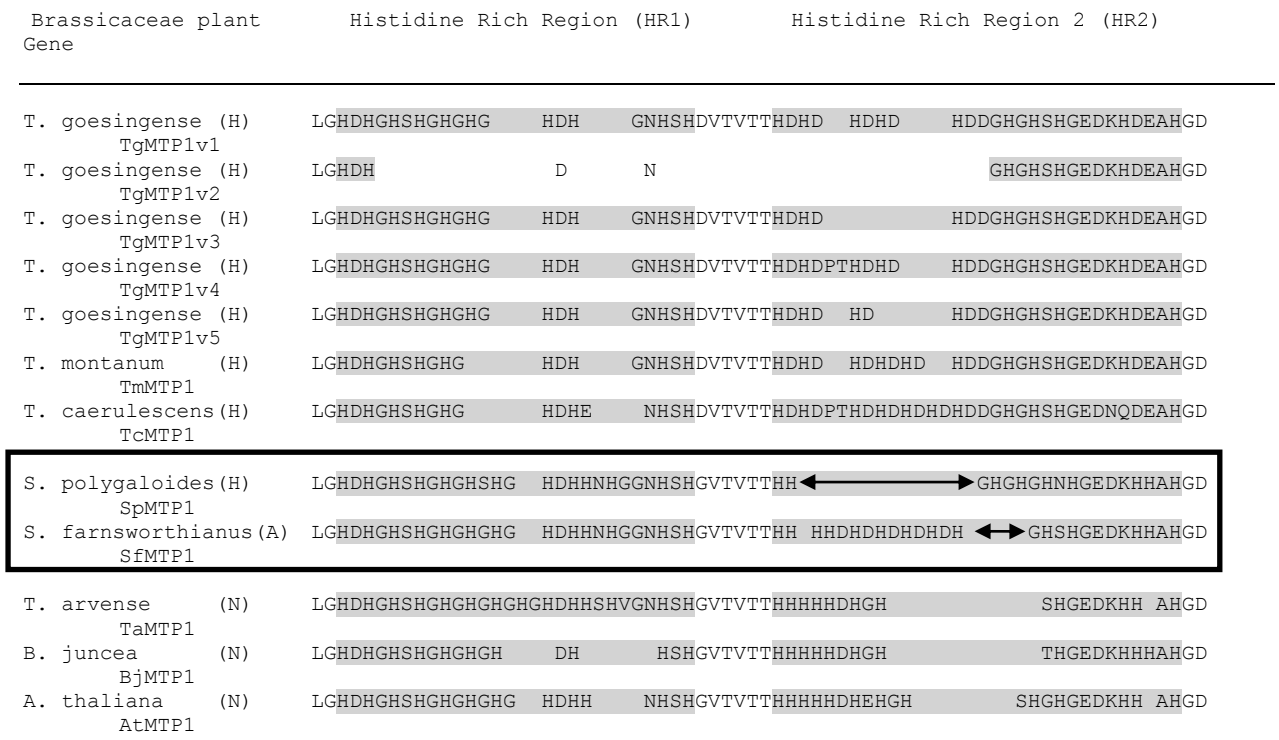


Figure 6. Comparison of the sequence alignments between the histidine rich regions of the MTP1 proteins found in metal hyperaccumulators (H), accumulator (A), and non-accumulator (N) plant species. The histidine-rich regions of *S. farnsworthianus* and *S. polygaloides* were compared to histidine rich regions of other hyperaccumulator and non-accumulator plant species derived from Persans *et al.* (2001) and Llemit (2005). A gap indicated with arrows.

Metal Tolerance Assays

To confirm the differences in metal tolerance conferred by *SfMTP1* and *SpMTP1*, these cDNAs were heterologously expressed in the metal sensitive yeast *Saccharomyces cerevisiae* strain *Δpep5::trp1*. This mutant yeast strain is metal sensitive, because it does not have normal sized vacuoles and possess abnormally small and dense vesicles (Woolford *et al*., 1990; Persans *et al*., 2001). Therefore, most of the MTP1 protein products are targeted to the plasma membrane (Kim *et al*., 2004). When the two genes were individually expressed in the yeast, metal tolerance varied depending on the metal the yeast was exposed to.

In the zinc and cobalt metal tolerance assays, there were no significant differences between metal tolerance conferred by the *S. farnsworthianus* and *S. polygaloides* genes in comparison to the control. *SfMTP1*, *SpMTP1*, *pYES2* showed similar levels of metal tolerance in the zinc assay. However, these averages seem to indicate that *SfMTP1* and *SpMTP1* cause the yeast to be somewhat more sensitive to zinc toxicity. Similar levels of metal tolerance were also seen in the cobalt metal tolerance assay.

Surprisingly, in the nickel metal tolerance assay *SfMTP1* had a higher Ni tolerance than the known Ni hyperaccumulator and the control. *SfMTP1* had a 2.13-fold higher Ni tolerance than the *pYES2* control and 1.63-fold higher Ni tolerance than *SpMTP1*. *SpMTP1* had a 1.30-fold higher Ni tolerance than the *pYES2* control.

The cadmium metal tolerance assay was the only assay supporting the hypothesized metal tolerance effect of both genes. In this assay, *SpMTP1* imparted the highest metal tolerance while *SfMTP1* imparted intermediate tolerance and the control showed little tolerance. *SpMTP1* had a 3.08-fold higher Cd tolerance than the *pYES2* control and a 1.77-fold higher Cd tolerance than *SfMTP1*. *SfMTP1* had a 1.72-fold higher Cd tolerance than the *pYES2* control.

Conclusion

The results of the metal tolerance assays show that the *S. farnsworthianus* and *S. polygaloides MTP1* cDNAs conferred increased metal tolerances to yeast exposed to Ni and Cd, while they showed a similar metal tolerance to the controls for Zn and Co.

The cadmium disk assay was the only metal tolerance assay meeting expectations. The hyperaccumulator cDNA, *SpMTP1*, conferred higher tolerance to the metal sensitive yeast than the *SfMTP1* and *pYES2*. The *SfMTP1* cDNA showed intermediate tolerance in comparison to the three genes. This was predicted because cadmium is highly toxic, particularly toward organisms that do not have the ability to tolerate metals and hyperaccumulators have more efficient strategies to detoxify toxic metals ions. The metal sensitive yeast strain with *SfMTP1* and *SpMTP1* reflect this trait.

The cobalt disk assay showed that *SfMTP1* and *SpMTP1* cDNAs did not confer Co tolerance to the yeast in comparison to the *pYES2* control. This implies that the SfMTP1 and SpMTP1 proteins may not be involved in the regulation of Co tolerance.

The metal accumulator cDNA, *SfMTP1*, imparted lower or similar metal tolerance in comparison to *SpMTP1* in all the metal disk assays except for Ni. In the Ni metal tolerance assay, *SfMTP1* showed the highest tolerance of the three genes. This was very surprising, because the *SpMTP1* cDNA comes from a known hyperaccumulator (Reeves *et al.,* 1983; Peer *et al*., 2006). Into (2010) and Y. Cerino (unpublished results), showed that *S. polygaloides* plants accumulate more Ni than *S. farnsworthianus* and enzymatic assays indicated that *S. farnsworthianus* should be categorized as a "metal tolerant non-accumulator plant species" (Into, 2010). The enzymatic assays performed by Into (2010) tested additional strategies metal accumulating plants use as a defense against toxic ions. Although these plant metal tolerance

genes were expressed in yeast, the genes do not necessarily act the same as they do in plants because of the heterologous expression environment (Kim *et al*., 2004).

The *pYES2* control responded as predicted for all disk assays except for the Zn metal tolerance assay. In the Zn disk assay, *pYES2*-containing yeast had a higher tolerance to Zn than the yeast with the *SfMTP1* and *SpMTP1* cDNAs. Meanwhile, *SfMTP1* and *SpMTP1* expressing yeast showed similar sensitivities to Zn when compared to each other. Even though statistical analysis showed that the results for the Zn metal tolerance assay were not significantly different, the averages of the zones of inhibition indicated that there may be a trend toward SfMTP1 and SpMTP1 proteins making the yeast more Zn sensitive. Additional testing is needed to verify that the control and metal accumulating genes are different.

Kim *et al*. (2004) shows that TgMTP1 (*Thlaspi goesingense* MTP1) protein is targeted to the plasma membrane when heterologously expressed in yeast and *A. thaliana*. Using this as a guideline, general trends from the results can be elucidated. To keep toxic ions from interfering with normal cell processes, *S.cerevisiae* will implement strategies in response to stress. One such strategy is the use of a proton gradient to mediate ion exchanges across the plasma membrane; a metal ATPase pump will push toxic metal cations back across the plasma membrane and bring in a hydrogen ion (Papoyan and Kochian, 2004). Transformation with *SfMTP1* and *SpMTP1* cDNAs in metal sensitive yeast indicates that the increased tolerance is possibly due to the Ni and Cd being effluxed out across the plasma membrane. There was no significant difference in metal tolerance conferred by *pYES2*, *SfMTP1*, or *SpMTP1* cDNAs to Co or Zn. Therefore it is probable that these MTP1 proteins may not transport Co out of the yeast cells. Further testing of the SfMTP1 and SpMTP1 protein expression is needed to confirm that these proteins impart Zn sensitivity. However, an explanation of this trend in Zn sensitivity

could be caused by increased Zn import into the cell by the MTP1 transporter. The accumulation of zinc inside the yeast cells would interfere with cellular processes causing lack of the yeast growth and increased zinc sensitivity.

The MTP1 proteins also can be targeted to the vacuolar membrane, where the metal ions will be sequestered into the vacuole to protect cellular components from damage (Persans *et al.*, 2001). Overexpression of MTP1 proteins from the GAL1 galactose-inducible promoter of *pYES2* can lead to their mislocalization and be targeted to the plasma membrane, vacuolar membrane or both (MacDiarmid *et al*., 2002; Kim *et al*., 2004).

Higher metal tolerance conferred by *SfMTP1* and *SpMTP1* cDNAs is likely explained by the efflux of a hydrogen ion from the internal compartment of the vacuole into the cytosol and the sequestering of Cd^{2+} or Ni^{2+} ion into the vacuole. A proton/cation antiporter will exchange a hydrogen ion for metal ions accumulating into the vacuole across a concentration gradient (Nishimura *et al.*, 1998; Gaxiola *et al.*, 2002). A similar yeast tolerance conferred by *pYES2*, *SfMTP1*, and *SpMTP1* cDNAS to Co, implies that MTP1 protein is not involved with Co transport. Likely trends for Zn sensitivity indicate that SfMTP1 and SpMTP1 proteins cause the proton/cation antiporter to push Zn out of the vacuole in exchange for a hydrogen ion. Thus zinc remains in the cytosol, and damage to essential cellular components results. Additionally, MTP1s proteins could target the improperly formed vacuoles. The MTP1 transporter may not work in the immature vacuolar membrane and ultimately hinder Zn accumulation in the vacuole.

S. cerevisiae can alter its membrane-based proton gradient to increase tolerance to toxic metal ions (Gaxiola *et al.*, 2002). To protect itself or tolerate metal, the yeast cells may utilize cation/proton transport methods to prevent excess metal ions in the cytosol because if there are more metal ions within the cell than the external environment of yeast, then the yeast cells will

be more sensitive to the metal. If there are fewer metals ions in the cytosol than outside of the cell, then the cell will be more metal tolerant. Equal amounts of metal ions inside and outside of the yeast will show no change in yeast sensitivity to the metal.

In comparing the alignment of the histidine-rich regions I and II of other Brassicaceae species to SfMTP1 and SpMTP1, the variable region can be identified. This region can be compared to *TgMTP1* (*Thlaspi goesingense*) which has already been expressed in *S. cerevisiae* (Persans *et al.*, 2001). It was shown that *TgMTP1* conferred Cd, Co, Zn, and Ni metal tolerance to yeast. However, *TgMTP1v1* conferred optimal Cd, Co, and Zn tolerance in relation to *TgMTP1v2* which conferred optimal Ni tolerance. According to Persans *et al*., (2001) gaps in the protein sequences may affect the level of Ni tolerance. In examining the alignment in Figure 6, the hyperaccumulator protein SpMTP1 shows a large gap in the second histidine-rich region. Increased Ni tolerance of yeast expressing *SpMTP1* is consistent with the literature. All hyperaccumulator species in Figure 6 showed gaps in the second histidine-rich region except for TcMTP1 (*Thlaspi caerulescens*) which showed increased HD duplications. The Ni disk assay showed that *SfMTP1* cDNAconferred higher Ni tolerance than *SpMTP1* cDNA. This was unexpected because the SfMTP1 protein sequence did not exhibit large gaps in the histidine-rich region but increased HD duplications in the same area. The other notable difference would be the five amino acid gap occurring after the duplications. This gap may be relevant because the same gap is seen in the non-accumulator plant species' protein sequences. Additionally, this gap may reflect metal specificity and transport abilities utilized by non-accumulator plant species. Also, only one allele was cloned for each species in this study. There may be other alleles that are specific for different types of metals (Persans *et al*., 2001).

Differential metal tolerance in the yeast may be due to a difference in protein gaps. *TgMTP1* cDNA was shown to confer Ni, Zn, Co, and Cd tolerance to yeast. *SfMTP1* and *SpMTP1* cDNAs conferred only Ni and Cd tolerance to *S. cerevisiae*. The variable histidine-rich regions among the MTP1s in these plant species may be conferring selective metal ion tolerance to the yeast cells.

Evaluation of *SfMTP1* and *SpMTP1* cDNA sequences are necessary to assess the metal tolerance advantages they may impart to transgenic *A. thaliana*. Transgenic *A. thaliana* plants discussed in the methods are currently being selected and grown to the third generation for further investigation. These plants will provide information on engineered Ni tolerance conferred to a known non-accumulator plant, *A. thaliana*. Since the transgenic plants did not show any aberrant growth or inability to produce viable seeds, the outcome of this experiment should be positive.

The yeast metal tolerance assays have provided evidence in how these genes will affect plant metal tolerance. However, the differences observed may result from different cell regulation mechanisms utilized between the yeast and plant cells. In addition, it is important to note that metal tolerance differences seen in transformed *S. cerevisiae* may not occur identically in transgenic plants. MTP1 proteins are regulated differently in plants and may be targeted to a different membrane than the yeast, have different trafficking strategies, be affected by different cofactors, or have a different metal specificity in the plants than in the yeast (Kim *et al.*, 2004). More testing would be needed to account for these variances.

To engineer an efficient phytoremediation plant, additional investigation of metal tolerance and uptake by different hyperaccumulator and metal accumulator genes is necessary. Biochemical and genetic studies of metal tolerance mechanisms involving efficient metal uptake

and vacuolar sequestration or plasma membrane efflux are crucial to construct a hyperaccumulator model and ultimately genetically engineer the ideal phytoremediation plant (Whiting *et al*., 2004). This study provides information on vacuolar and plasma membrane mediated metal tolerance via the *SfMTP1* and *SpMTP1* cDNAs from *S. farnsworthianus* and *S. polygaloides.* Evidence indicates that the *SfMTP1* and *SpMTP1* cDNAs conferred differential metal tolerance to *S. cerevisiae* and that the plant genes may have different metal specificities concerning the transport of Ni, Cd, Co, and Zn metals.

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BIOGRAPHICAL SKETCH

Elyssa Rae Garza received her high school diploma in May of 2006 from Mission High School. After acceptance to the University of Texas-Pan American (UTPA), she pursued a degree in Biology and obtained her Bachelor's Degree of Science in December 2009. Immediately, the following Spring, she was accepted into the graduate program for the Department of Biology at the University of Texas-Pan American. During her time as a Master's candidate, Ms. Garza worked as a Math Tutor and Graduate Assistant for Anatomy and Physiology I laboratory while pursuing her research in plant molecular biology. She was also the treasurer for Latinos in Science and Engineering (MAES) UTPA chapter. After defending her thesis in July 2013, she earned her Master's Degree of Science in Biology. Currently, she was accepted to Texas A&M University at College Station and is pursuing a doctorate in Genetics. She can be reached at her permanent mailing address: 1000 Adams St., Mission, TX, 78572.