UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigation of *Arabidopsis thaliana* Mutants with Altered Heavy Metal Induced Gene Expression

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in

Biology

by

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The Thesis of Yasman Zarabi is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ACKNOWLEDGEMENTS

To my incredibly loving family, you have loved me selflessly, and all of your sacrifices have been taken to ensure the betterment of my life and my future. The gratitude I feel, words could not express for all you have done.

I want to thank my principal investigator Julian Schroeder for taking the time to establish the foundation for my technical laboratory skill set that I will carry with me into my future endeavors. Thank you for all of your ongoing support and care, both academically and in my personal endeavors.

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ABSTRACT OF THE THESIS

Investigation of *Arabidopsis thaliana* Mutants with Altered Heavy Metal Induced Gene Expression

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Professor Julian Schroeder, Chair

Consumption of food crops with heavy metal and metalloid content may be associated with harmful health risks. After exposure to heavy metals, plants induce significant gene expression changes, but the mechanisms underlying this transcriptional response are unknown. Forward genetic screens for mutants displaying altered heavy metal-induced gene expression were performed to address this issue. Subsequent mapping of the causal genes provides an approach to illuminate the underlying mechanisms. “Luciferase reporter lines” were created by merging the promoter of cadmium-induced *SULTR1;2* gene to the bioluminescent firefly luciferase gene within *Arabidopsis thaliana* (Jobe et al., 2012). Mutant groups were created by subjecting “luciferase reporter lines” to ethyl methanesulfonate (Jobe et al., 2012). Forward genetic luciferase screens of mutagenized lines showed a phenotypic response when exposed to cadmium. Three mutant subgroups were defined as “constitutive response to cadmium (*crc1*)”, “super response to cadmium (*src1*)”, and “non-response & reduced response to cadmium (*nrc1,2* were characterized)” (Jobe et al., 2012). Evaluation of bulk segregation analysis led to the rough mapping of the cadmium-induced luciferase luminescence mutants *crc1* and *src1*. Genes in candidate regions were mapped, and their respective T-DNA insertion

lines were ordered. ICP-OES studies displayed no significant distinction in cadmium accumulation between wild-type, *crc1*, and *src1* mutants. Using RT-qPCR, *src1* mutants and candidate T-DNA insertion lines displayed similar transcriptional gene expression responses to cadmium. These results may expand our knowledge of plant genes involved in the heavy metal response.

CHAPTER 1 Investigation of *Arabidopsis thaliana* Mutants with Altered Heavy Metal Induced Gene Expression

Introduction

The substance water is vital and critical for the viability of plant and human survival. Plants uptake water through their roots from the encircling soil for survival and fulfill the physiological need to provide oxygen and nutrition essential to human survival. Human intake of plant material and ingesting drinking water containing heavy metals and some metalloids in our bodies can be hazardous to human health (Järup, 2003, Khan et al., 2015, Pujari & Kapoor, 2021). Heavy metals have also been associated with various oxidative stress-induced damage, leading to human disease (Gallego et al., 1996, Paithankar et al., 2021).

Heavy metals in the environment can be found naturally or accumulate in contaminated soils through mining workplaces and farming methods (Zhang & Wang, 2020). Abandoned mining sites pose a potential adverse health effect by ingestion or physical exposure to heavy metal contamination (Fashola et al., 2016, Moon et al., 2021). Eruption of volcanoes may leave trace levels of heavy metals toxic elements that may be linked to thyroid cancer (Vigneri et al., 2017, Malandrino et al., 2020). Dust was investigated as a way to come into contact with heavy metal contamination from possibly the nearby battery factories (Shen et al., 2021). Soil pollution due to agricultural practices may pose a human health risk (Zwolak et al., 2019, Zhang et al., 2021).

Selenium has the particular ambivalent function of being both beneficial and potentially harmful to human health. Selenium can be obtained by humans through diet and can have a positive functional effect on the immune and genital systems (Hossain et al., 2021). It was determined that the intake of selenium had a positive impact on combating oxidative damage, which may have been caused by exposure to the heavy metal cadmium in mice (Su et al., 2021). The metalloid selenium above required levels may induce toxicity (Spallholz, 1994, MacFarquhar, 2010). Cadmium can transfer to crops through soil pollution and may be harmful to human health (Kubier et al., 2019, Ma et al., 2021). Cadmium may be considered

carcinogenic and is known to affect red blood cells and cause damage to parts of the renal system (Satarug, 2018, Peters et al., 2021, Shi et al., 2021). Mother's breast milk contaminated with heavy metal can be passed along to the infant with the possibility of adverse health effects (Huat et al., 1983, Pajewska-Szmyt et al., 2019, Al-Saleh, 2021).

Many different organizations work together domestically and internationally to set requirements and recommendations when determining the appropriate concentrations of different elements for consumption, including possible tolerable heavy metals and metalloids. The U.S. Food and Drug administration contemplates numerous factors when considering the mechanism of exposure to heavy metals and sets guideline standards and recommendations for consumption and use. Collectively the World Health Organization (WHO) and several other organizations establish guidelines for contaminations in an effort to avoid adverse health implications when considering heavy metal pollution.

Glutathione is a molecule found in humans and plants and often is considered to be an antioxidant protecting against oxidation and the possible creation of toxic free radicals (Pompella et al., 2003, Anjum et al., 2011, Hasanuzzaman et al., 2017). Phytochelatins are assembled from glutathione in response to the presence of cadmium (Clemens et al., 1999, Sanità di Toppi & Gabbrielli, 1999, Cobbett, 2000). Phytochelatins are assembled, and crafted oligomers of glutathione in plants and have the possibility to function as a chelator that binds tightly to metal ions to help detoxify heavy metals (Clemens, 2006, Hasanuzzaman et al., 2017). Glutathione had been regarded and exhibited to reduce the stress on maize seedlings when exposed to cadmium (Wang et al., 2021). Metallothionines are proteins with an excess density of cysteine residues and an area of research regarding binding metals (Cobbett & Goldsbrough, 2002). The ability of metallothionines to bind metal ions in plants has the possibility to aid with heavy metal tolerance (Zimeri et al., 2005, Saeed-ur-Rahman et al., 2020).

Heavy metal exposure can be toxic for plants and can be acquired through soil (Nagajyoti et al., 2010). Heavy metals exposure in plants may cause a transcriptional change in

gene expression (Gratão et al., 2005, Wojas et al., 2008, Hasan et al., 2017). However, the underlying transcriptional control mechanisms are not well understood. In the presence of cadmium within *Arabidopsis thaliana*, a decrease in glutathione and other thiol concentrations and an oxidized state is needed to promote gene expression of the sulfate assimilation pathway (Jobe et al., 2012). Cadmium toxicity may possibly contribute to plant development by affecting gene regulation (Asgher et al., 2015, Benavides et al., 2005, Qin et al., 2020).

As introduced earlier, the transcription factors and transcriptional control mechanisms mediating cadmium-induced gene expression are not well understood. The transcriptional mechanism triggered by cadmium exposure of plants needs to be further studied to discover genes involved in the activation of heavy metal and cadmium-induced gene expression. A visible screen was done by using mutagenized *Arabidopsis thaliana* containing a reporter for cadmium-induced gene expression (Jobe et al., 2012). The “luciferase reporter lines” were created by merging the cadmium-induced *SULTR1;2* promoter gene with the bioluminescent firefly luciferase gene (Jobe et al., 2012). “Luciferase reporter lines” were screened to confirm the promoter region's insertion, and mutations were made by subjecting seeds to ethyl methanesulfonate (Jobe et al., 2012). Jobe et al. (2012) carried out a forward genetic screen for mutant lines with distinct phenotypes in the presence of cadmium (Cd) (Jobe et al., 2012). Three mutant subgroups were discovered and named as follows: mutant “constitutive response to cadmium (*crc1*)”, mutant “super response to cadmium (*src1*)”, and “non-response & reduced response to cadmium (*nrc1,2*)” (Jobe et al., 2012).

Further experiments were conducted to identify possible negative regulators of cadmium-induced gene expression by investigating mutants showing increased *SULTR1;2* gene expression. The previous screen conducted led to the subgroup of the mutant lines, “constitutive response to cadmium (*crc1*)” and “super response to cadmium (*src1*)” (Jobe et al., 2012). In this thesis, I have further characterized these mutants and advanced the testing of candidate genes identified previously by rough mapping of the *crc1* and *src1* loci.

Results

Previously, “luciferase reporter lines” were created by merging the promoter region of cadmium-induced *SULTR1;2* genes with the bioluminescent firefly luciferase gene (Jobe et al., 2012). Following confirmation, the wild-type “luciferase reporter lines” were subjected to ethyl methanesulfonate (EMS) mutagenesis to create mutant groups for forward genetic screening (Jobe et al., 2012). An observable phenotypic gene expression response to cadmium (Cd) was established. Mutant “constitutive response to cadmium (*crc1*)” and mutant “super response to cadmium (*src1*)” were two subgroups (Jobe et al., 2012). Andrew Cooper conducted bulk segregation analysis, which led to rough mapping and a way to identify mutations associated with cadmium-induced mutant *crc1* and *src1* phenotypes (Cooper, 2018). The bulk segregation analysis discovered 14 *crc1* and 25 *scr1* candidate mutations on chromosome 4 within the corresponding mapping areas of the *Arabidopsis thaliana* genomes of these two mutants (Cooper, 2018). The tables can be found in the dissertation of Andrew Cooper (Cooper, 2018). T-DNA insertion knockout lines for mutant *crc1* and *src1* candidate genes were ordered to identify causative mutations, and tables can be found in the dissertation of Andrew Cooper (Cooper, 2018).

1.1 Mutant “Constitutive Response to Cadmium *(crc1)”* (Jobe et al., 2012)

Previously, mutant *crc1* had a constitutive bioluminescent luciferase-induced response to cadmium and the absences of cadmium compared to “luciferase reporter lines” (WT) (Jobe et al., 2012). Upon starting this project, several mutant *crc1* lines displayed substandard growth and seed germination. For this reason, several mutant *crc1* lines were bulk grown, and plant seedlings that were found to have adequate germination were further propagated.
Mutant *crc1* lines were plated and grown on sulfur-free media for approximately 10-14 days, followed by plant growth protocol found in the material and method section. Luciferase assays

were done to confirm that the luciferase reporter (WT), mutant *crc1*, and *src1* lines had the presence of a functional luciferase reporter (Figure 1). Luciferase assays were conducted with and without the presence of 100μM of cadmium using ¼ MS with sucrose media. Growing conditions were followed using the protocol for luciferase assay in the material and method section. However, the level of bioluminescence in luciferase reporter (WT) treated with 100μM cadmium was not as strongly pronounced as previously observed (Figure 1A and 1B, Jobe et al., 2012). In the *crc1* mutant, the level of bioluminescence with and without 100μM cadmium was not strongly pronounced as previously observed for both conditions (Figure 1C and 1D, Jobe et al., 2012). The level of bioluminescence in *src1* mutant treated with 100μM cadmium was not strongly pronounced as previously observed (Figure 1E and 1F, Jobe et al., 2012). Possibly gene silencing may have taken place for the luciferase reporter through bulking and propagation of lines.

To measure cadmium accumulation, Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) experiments were conducted for luciferase reporter (WT), mutant *crc1*, and *src1* lines. Three replicate plates of whole plant seedlings for each sample line were plated and grown for approximately 14 days and either collected or taken to 100μM of cadmium for approximately 6 hours and 100μM of cadmium for approximately 72 hours (Figure 2, Figure 3). Growing conditions were followed using the protocol for inductively coupled plasma-optical emission spectrometry in the material and method section. All lines, compared to themselves at the non-cadmium condition, 100μM of cadmium for 6 hours and 100μM of cadmium for 72 hours showed a significant notable gradual increase in cadmium accumulation, respectively (Figure 2, Figure 3). Compared to each other, all lines were found to have similar cadmium accumulation in the non-cadmium condition, 100μM cadmium for 6 hours and 100μM cadmium for 72 hours (Figure 2, Figure 3). Wild-type luciferase reporter (WT), mutant *crc1*, and *src1* lines compared to each other at the same concentrations and for the same time duration were found to have no significant difference in cadmium accumulation (Figure 2, Figure 3).

Previously, mutant *crc1* lines were noted to exhibit an extended root length in 75μM cadmium with 1 μM of selenium compared to wild-type luciferase reporter (WT) (Cooper, 2018). CRC SALK_T-DNA insertion lines that exhibited similar extended root length as those once discovered for mutant *crc1* were recognized to be T-DNA insertion lines 067394 and 120184 (gene *At4g10930*) and T-DNA insertion lines 117073 and 117071 (gene *At4g13575*) through previously conducted root growth assays by Andrew Cooper (Cooper, 2018). Based on this data and previous root growth assays, I conducted root-growth assays at 55μM, 75μM and 100μM of cadmium with and without selenium for luciferase reporter (WT), mutant *crc1*, and T-DNA insertion CRC lines 067394 (gene *AT4G10930)*, 120184 (gene *AT4G10930*), and 117073 (gene *AT4G13575*) (Figure 4 (55 μM condition), Figure 5 (75 μM condition), Figure 6 (100 μM condition)). The root growth assays were grown according to the protocol for root growth assay found in the material and method section. Three replicate plates for each treatment condition were plated and grown on sulfur-free media for approximately 7 days and then taken to either control sulfur-free media or 55μM, 75μM, and 100μM cadmium, or 55μM, 75μM, and 100μM cadmium with 1μM of selenium for another approximately 7 days (Figure 4 (55 μM of cadmium), Figure 5 (75 μM of cadmium), Figure 6 (100 μM of cadmium)).

Mutant *crc1* lines did not display an average extended root length in 75μM cadmium with 1μM of selenium compared to wild-type luciferase reporter (WT) as previously observed (Figure 5). The difference in root growth between genotypes was within one standard deviation so that no firm conclusion can be drawn. The average root length for all genotypes had inhibited root length in 100μM cadmium condition (Figure 6). The difference in root growth between genotypes was within one standard deviation so that no firm conclusion can be drawn. It was observed that T-DNA CRC insertion lines 067394 (gene *AT4G10930)* had an extended average root length in 100μM cadmium with 1μM selenium when compared to cadmium without selenium (Figure 6). The difference in root growth between genotypes was within one standard deviation so that no firm conclusion can be drawn.

1.2 Mutant “Super Response to Cadmium (*src1*)” (Jobe et al., 2012)

Previously, mutant *src1* lines had a super bioluminescent luciferase-inducted response to cadmium compared to the wild-type luciferase reporter (WT) (Jobe et al., 2012). The following genes were previously found to have increased SULTR1;2 gene expression within cadmium similar to mutant *src1* exhibited by brighter intensity bands through RT-PCR when compared to luciferase reporter (WT), gene *AT4G16267*, and gene *AT4G15230* found in the thesis of Alexander Scavo (Scavo, 2019). Based on this data and previous RT-PCR, I pursued experiments to investigate candidate genes using Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR).

To assess candidate mutants for the mutant *src1* phenotype, a Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) was used to examine the quantitative transcriptional gene response to cadmium. RT-qPCR experiments were conducted for luciferase reporter (WT), mutant *src1*, and SRC SALK_T-DNA insertion candidate lines 061827 and 021343 (pseudogene *AT4G16267, and gene AT4G16270*) (Figure 9). Gene *AT4G16270 and* pseudogene *AT4G16267* are on opposite strands and have overlapping regions (Figure 10) (https://www.arabidopsis.org/, http://signal.salk.edu). Pseudogene *AT4G16267*, according to The Arabidopsis Information Resource (TAIR), is transcribed and translated to a plant thionin (https://www.arabidopsis.org/, Lamesch et al., 2012). Gene *AT4G16270,* according to TAIR, is transcribed and translated to a class III peroxidase (https://www.arabidopsis.org/, Lamesch et al., 2012). Plant seedlings were plated and grown on ¼ MS media for approximately seven days. The plant seedlings were then taken to either control ¼ MS media plates with no cadmium or ¼ MS media with 100µM of cadmium for approximately six hours before extraction of total RNA and cDNA synthesis. RNA extracted sample values were measured using a nanodrop spectrophotometer prior to running RT-qPCR (Table 1, Figure 7). cDNA extracted sample values were measured using a nanodrop spectrophotometer prior to running RT-qPCR (Table 2, Figure 8). RT-qPCR was performed and measured the expression level of cadmium-inducible

gene *AT1G78000.*1 (SULTR1;2), and housekeeping gene *AT5G60390* (Ef1a) was used. The average fold change values included 3 biological samples for all lines, except for the 061827 control gene of interest, with 2 biological samples due to technical issues.

Luciferase reporter (WT) had no notable average fold change in SULTR1;2 gene expression without cadmium compared to luciferase reporter (WT) with 100 μM cadmium (Figure 9). Note that RT-qPCR assays differ from luciferase reporter assays. Thus, this result provided a baseline for comparison to the same treatment in other SRC SALK_T-DNA insertion lines. Mutant *src1* had a notable increase in average fold change in SULTR1;2 gene expression with 100μM cadmium compared to mutant *src1* without cadmium (Figure 9). SALK_T-DNA insertion line 061827 had a notable increase in average fold change in SULTR1;2 gene expression with 100μM cadmium compared to SALK_T- DNA line 061827 without cadmium (Figure 9). SALK_T-DNA insertion line 021343 had a notable increase in average fold change in SULTR1;2 gene expression with 100μM cadmium compared to T-DNA line 021343 without cadmium (Figure 9). T-DNA insertion lines 021343 and 061827 (pseudogene *AT4G16267* and gene *AT4G16270)* both displayed increased SULTR1;2 gene expression in the presence of cadmium, similar to mutant *src1* compared to their respective lines without cadmium (Figure 9).

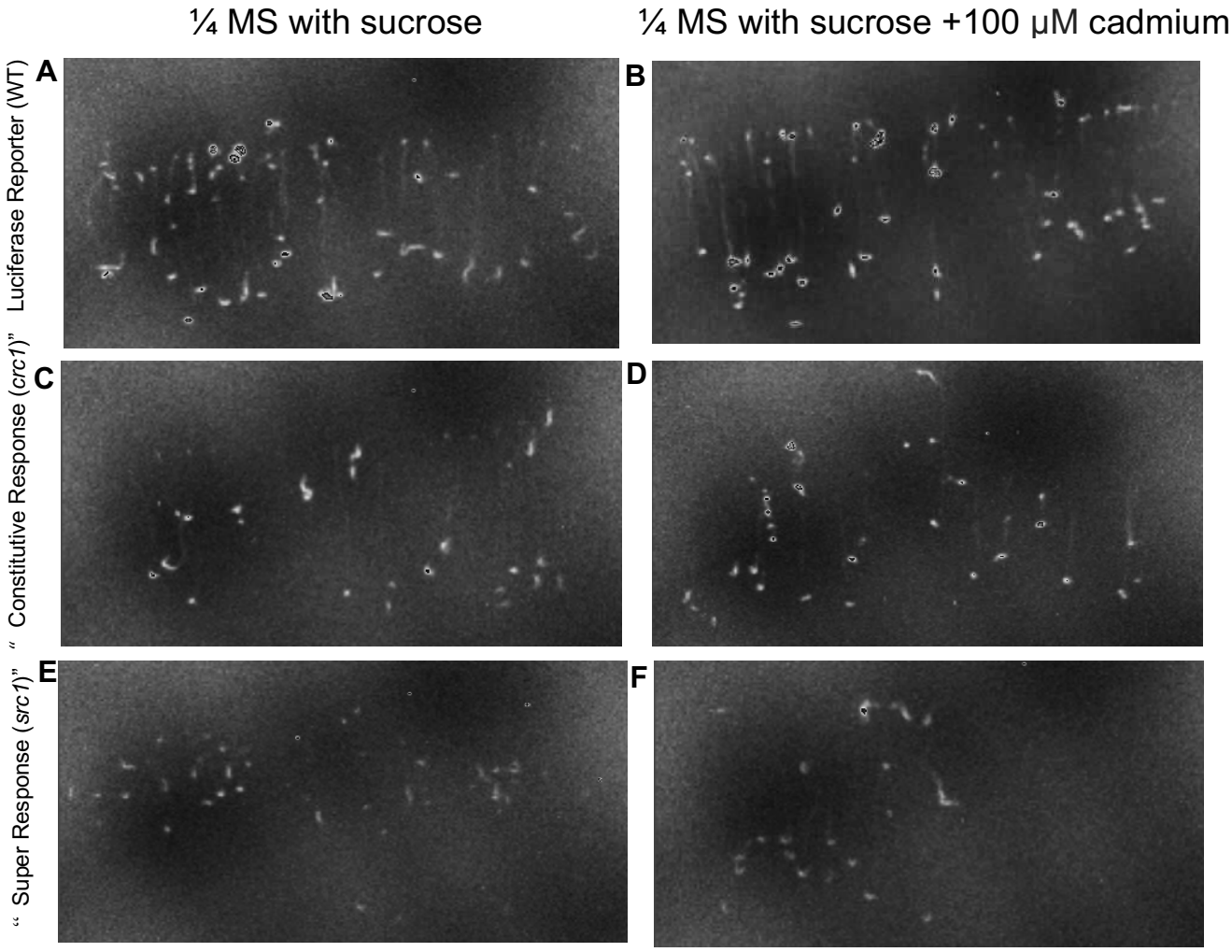

Figure 1: Luciferase Assay response experiments were conducted on wild-type "luciferase reporter line" (WT), mutants "constitutive response to cadmium (*crc1*)", and "super response to cadmium (*src1*)" lines. Lines were plated and grown on ¼ MS with sucrose media, and 100 µM of luciferin was used. Images were taken after approximately 6 hours of exposure to either ¼ MS with sucrose media plates or ¼ MS with sucrose media plates with 100 µM cadmium. WinLight Berthold Technologies imaging software was used. Contrast & Brightness set the same for all images in PowerPoint. **A**) Luciferase reporter (WT) no cadmium **B**) Luciferase reporter (WT) with 100 µM cadmium **C**) *crc1* no cadmium, **D**) *crc1* with 100 µM cadmium **E**) *src1* no cadmium **F**) *src1* with 100 µM cadmium.

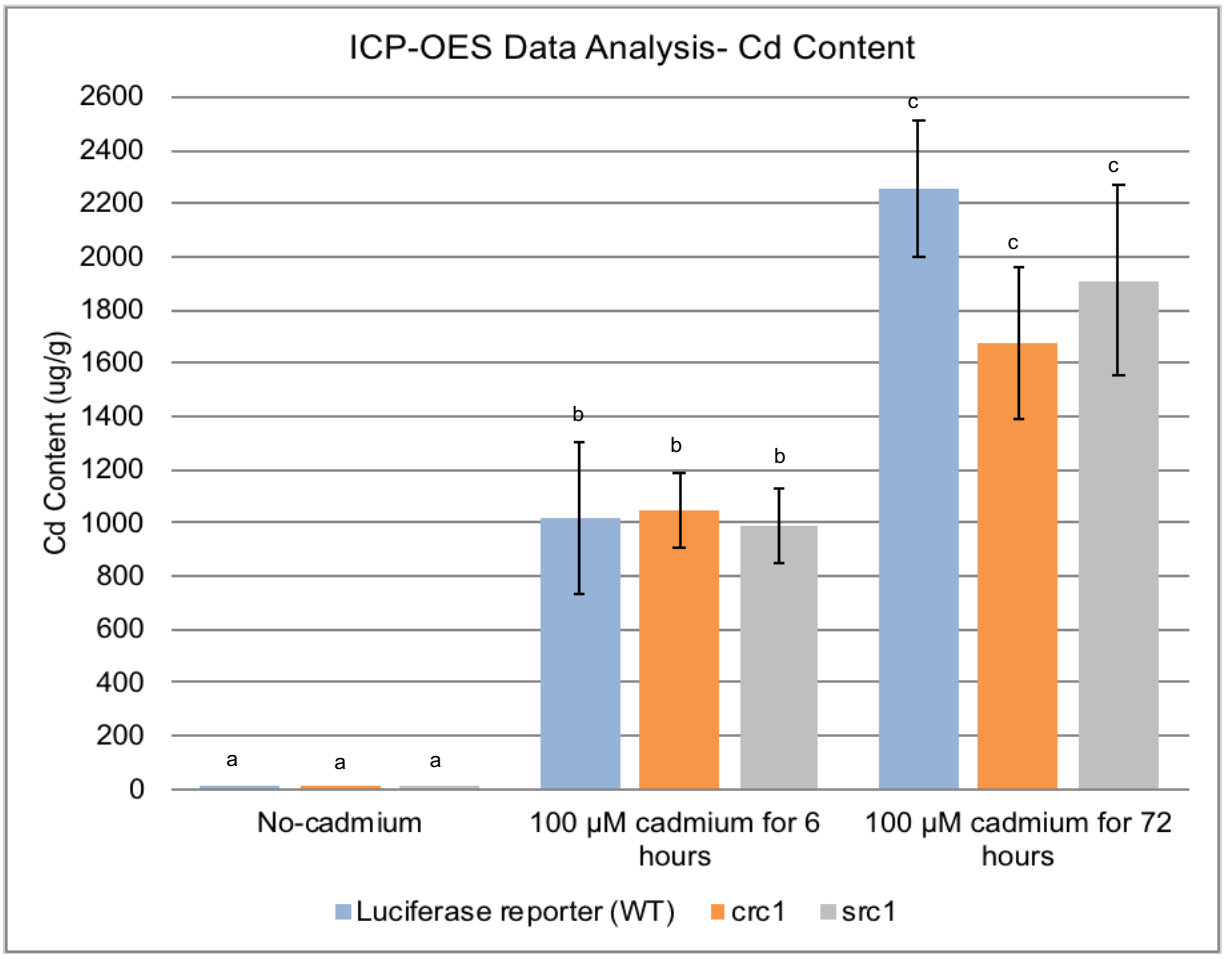

Figure 2: To measure cadmium content, Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) experiments were conducted for "luciferase reporter line" (WT), "constitutive response to cadmium (*crc1*)", and "super response to cadmium (*src1*)" lines on sulfur-free media. Three replicate plates of whole plant seedlings for each sample line were plated and grown for 14 days and collected or taken to 100 µM of cadmium for 6 hours and 100 µM of cadmium for 72 hours. The error bars in this figure display standard deviation. The one-way ANOVA analysis and Tukey HSD test resulted in p-values when evaluating the three genotypes under one condition: a) 0.52 b) 0.94 c) 0.14 and were found not statistically significant between genotypes. A cut-off value of $p < 0.05$ would indicate a >95% confidence interval which was not found when comparing the three genotypes within each condition.

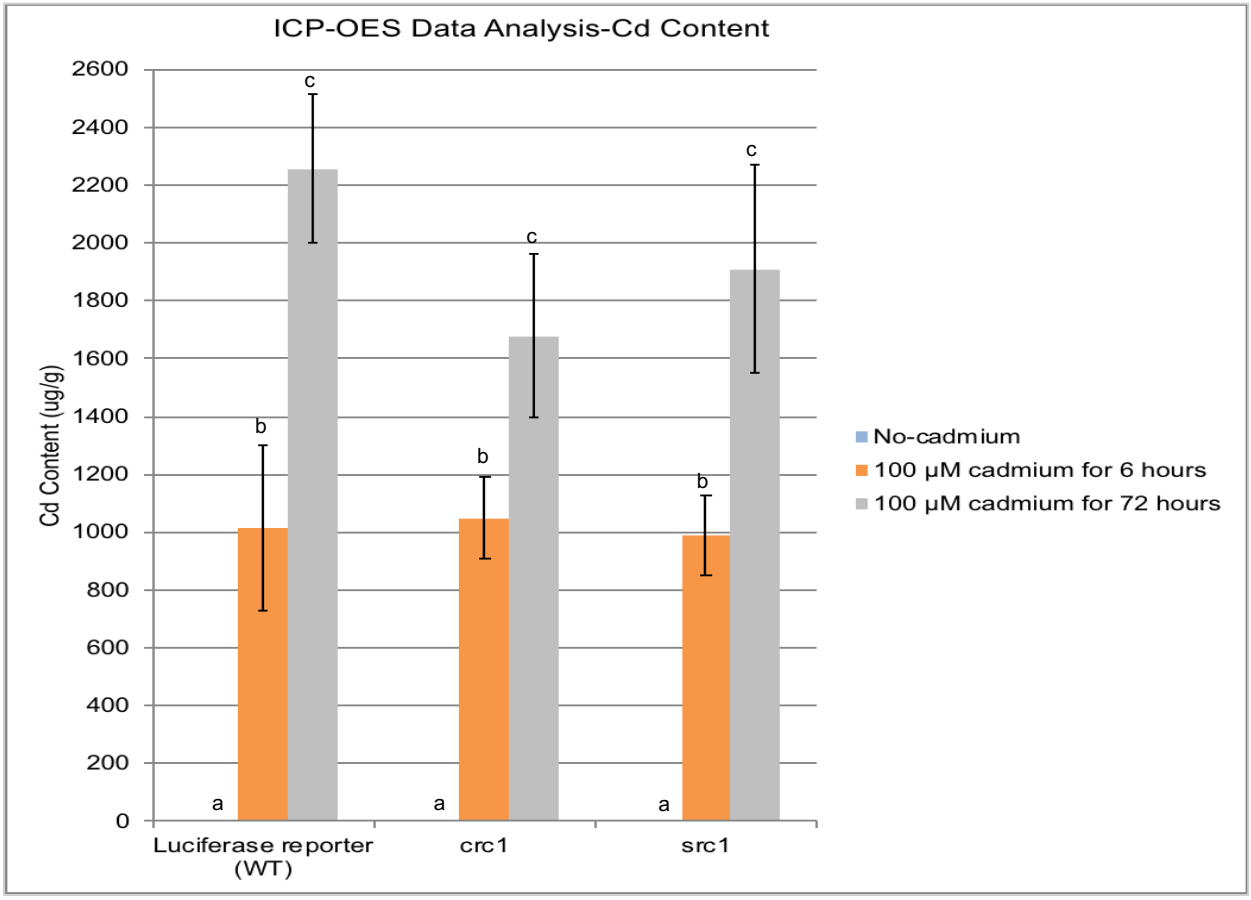

Figure 3: To measure cadmium content, Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) experiments were conducted for "luciferase reporter line" (WT), "constitutive response to cadmium (*crc1*)", and "super response to cadmium (*src1*)" lines on sulfur-free media. Same data used from Figure 2, different visual representation. Three replicate plates of whole plant seedlings for each sample line were plated and grown for 14 days and collected or taken to 100 µM of cadmium for 6 hours and 100 µM of cadmium for 72 hours. The error bars in this figure display standard deviation. The one-way ANOVA analysis and Tukey HSD test resulted in p-values when evaluating the three genotypes under one condition: a) 0.52 b) 0.94 c) 0.14 and were found not statistically significant between genotypes. A cut-off value of $p < 0.05$ would indicate a >95% confidence interval which was not found when comparing the three genotypes within each condition.

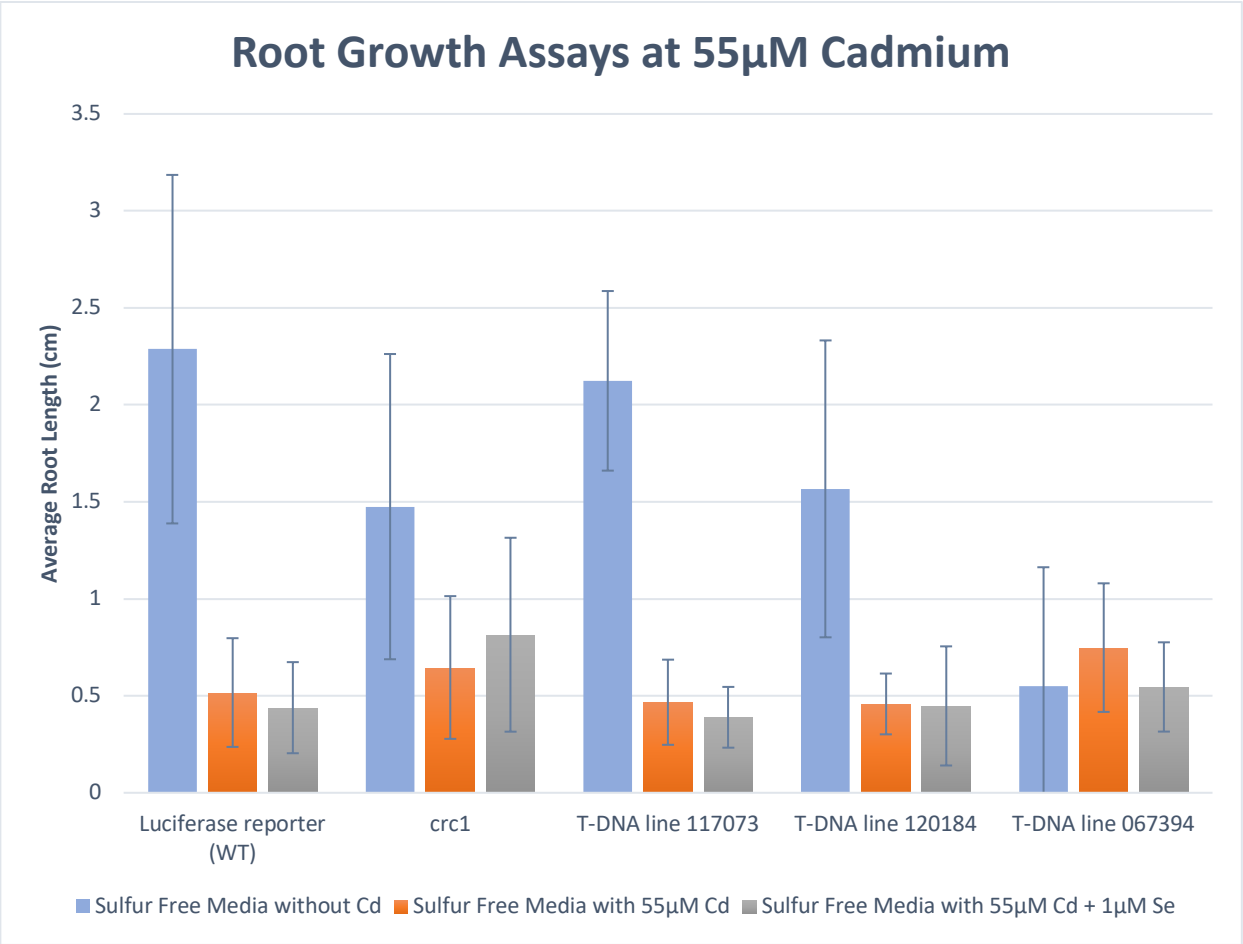

Figure 4: Plant root growth assays were analyzed for luciferase reporter (WT), mutant "constitutive response to cadmium (*crc1*)", and mutants in candidate CRC genes via T-DNA insertion SALK_ lines 117073 (gene *AT4G13575*), 120184 (gene *AT4G10930*), and 067394 (gene *AT4G10930*). Three replicate plates for each treatment condition were plated and grown on sulfur-free media for approximately 7 days and then taken to either control sulfur-free media and 55 µM cadmium and 55 µM cadmium with 1 µM Se for approximately another 7 days of growth. Error bars in this figure display standard deviation. Approximately 12 seedlings per line for three plates were grown and were analyzed.

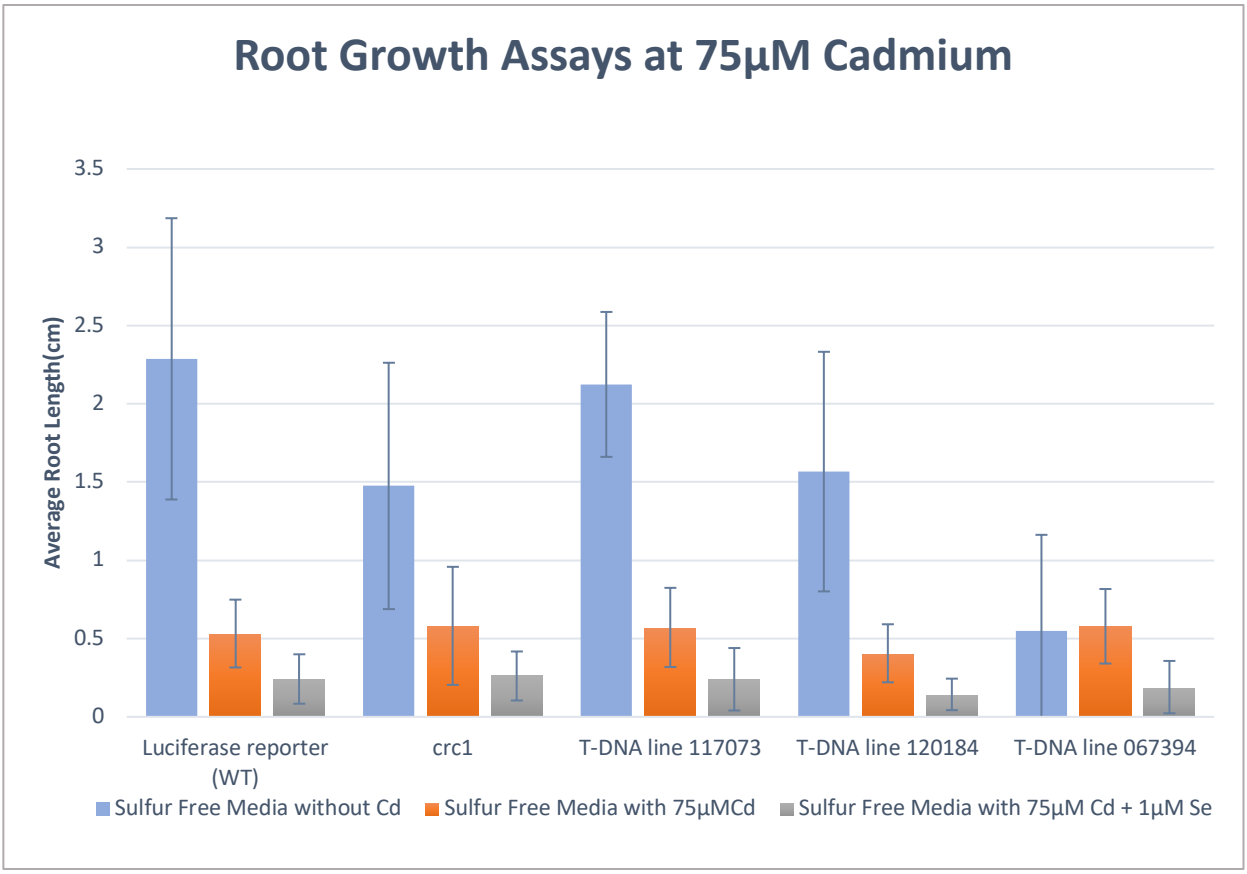

Figure 5: Plant root growth assays were analyzed for luciferase reporter (WT), mutant "constitutive response to cadmium (*crc1*)", and mutants in candidate CRC genes via T-DNA insertion SALK_ lines 117073 (gene *AT4G13575*), 120184 (gene *AT4G10930*), and 067394 (gene *AT4G10930*). Three replicate plates for each treatment condition were plated and grown on sulfur-free media for approximately 7 days and then taken to either control sulfur-free media and 75 µM cadmium and 75 µM cadmium with 1 µM Se for another approximately 7 days of growth. Error bars in this figure display standard deviation. Approximately 12 seedlings per line for three plates were grown and were analyzed.

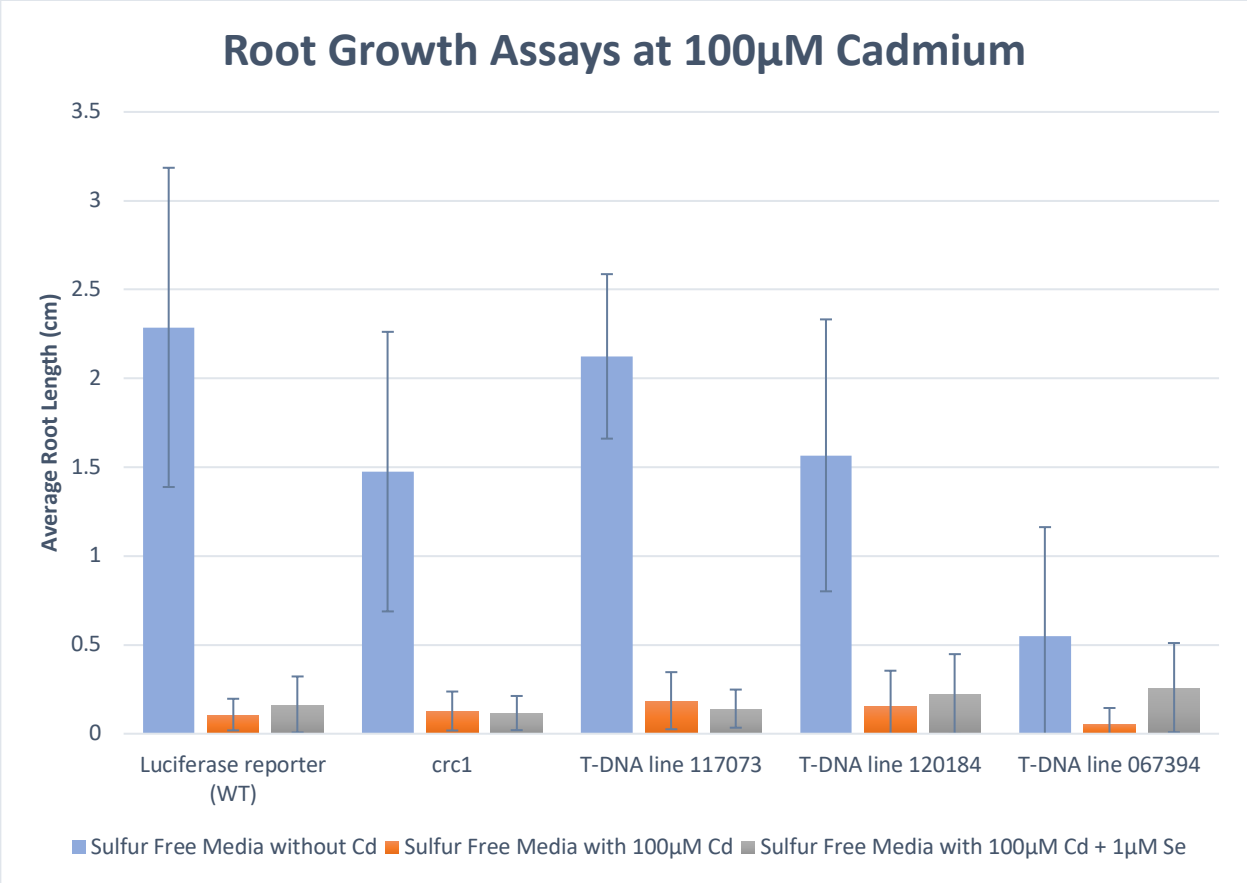

Figure 6: Plant root growth assays were analyzed for luciferase reporter (WT), mutant "constitutive response to cadmium (*crc1*)", and mutants in candidate CRC genes via T-DNA insertion SALK_ lines: 117073 (gene *AT4G13575*), 120184 (gene *AT4G10930*), and 067394 (gene *AT4G10930*). Three replicate plates for each treatment condition were plated and grown on sulfur-free media for approximately 7 days and then taken to either control sulfur-free media and 100 µM cadmium and 100 µM cadmium with 1 µM Se for another approximately 7 days of growth. Error bars in this figure display standard deviation. Approximately 12 seedlings per line for three plates were grown and were analyzed.

Table 1: RNA purification values were taken with a nanodrop spectrophotometer prior to RT-qPCR. Units measured were ng/ul concentration, 260/280 ratio, and 260/230 ratio in determining samples.

Lines	ng/ul	260/280	260/230
SRC SALK_T-DNA Line 061827	554.1 ng/ul	2.10	2.34
SRC SALK_T-DNA Line 061827 +100 µM of Cadmium	577.7 ng/ul	2.08	2.17
SRC SALK_T-DNA Line 021343	553.5 ng/ul	2.08	2.33
SRC SALK_T-DNA Line 021343 + 100µM of Cadmium	704.2 ng/ul	2.10	2.33
Luciferase reporter control (WT)	664.8 ng/ul	1.97	1.55
Luciferase reporter control (WT) + 100 µM of Cadmium	451.6 ng/ul	2.06	2.24
src1	747.0 ng/ul	2.12	2.35
src1 + 100 µM of Cadmium	781.5 ng/ul	2.11	2.36

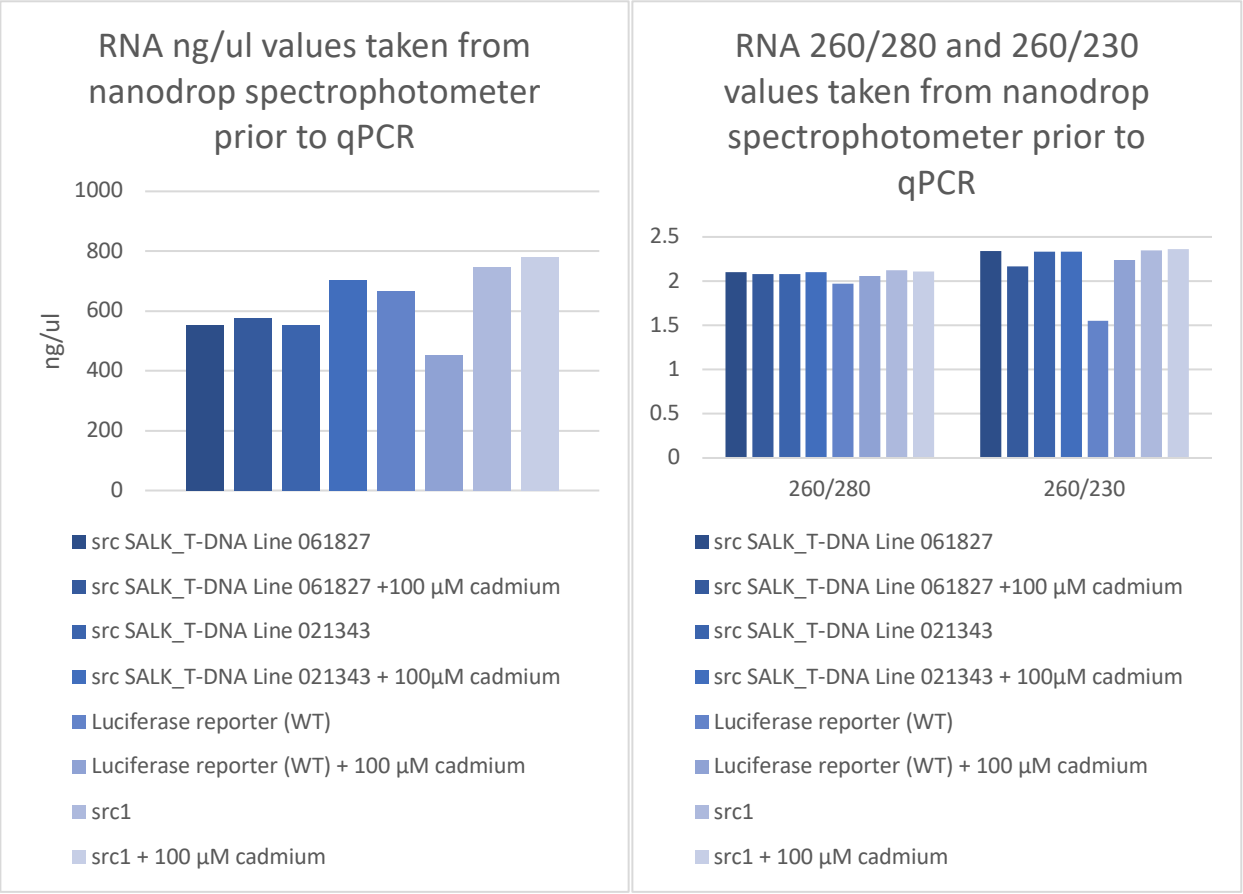

Figure 7: RNA purification values were taken with a nanodrop spectrophotometer prior to RT-qPCR. Units measured were ng/ul concentration, 260/280 ratio, and 260/230 ratio in determining samples. Same data used from Table 1, different visual representation.

Table 2: cDNA purification values were taken with a nanodrop spectrophotometer prior to RT-qPCR. Units measured were ng/ul concentration, 260/280 ratio, and 260/230 ratio in determining samples.

Lines	ng/ul	260/280	260/230
SRC SALK_T-DNA Line 061827	3844.6 ng/ul	1.69	1.20
SRC SALK_T-DNA Line 061827 +100 µM of Cadmium	4067.0 ng/ul	1.73	1.92
SRC SALK_T-DNA Line 021343	4054.3 ng/ul	1.73	1.83
SRC SALK_T-DNA Line 021343 + 100µM of Cadmium	4644.0 ng/ul	1.72	1.87
Luciferase reporter control (WT)	4376.6 ng/ul	1.73	1.87
Luciferase reporter control (WT) + 100 µM of Cadmium	3666.0 ng/ul	1.72	1.80
src1	4971.2 ng/ul	1.69	1.38
src1 + 100 µM of Cadmium	4312.2 ng/ul	1.71	1.47

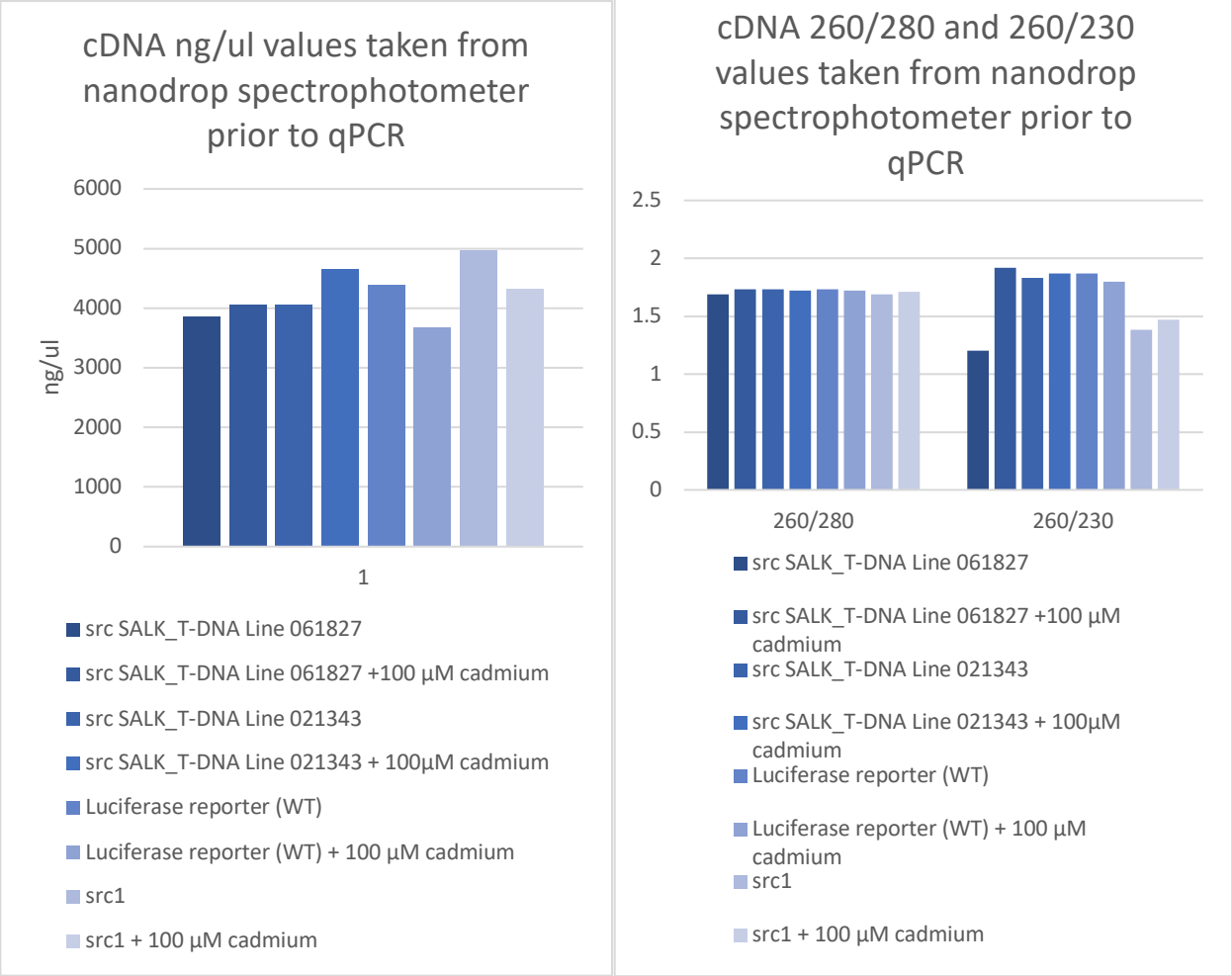

Figure 8: cDNA purification values were taken with a nanodrop spectrophotometer prior to RT-qPCR. Units measured were ng/ul concentration, 260/280 ratio, and 260/230 ratio in determining samples. Same data used from Table 2, different visual representation.

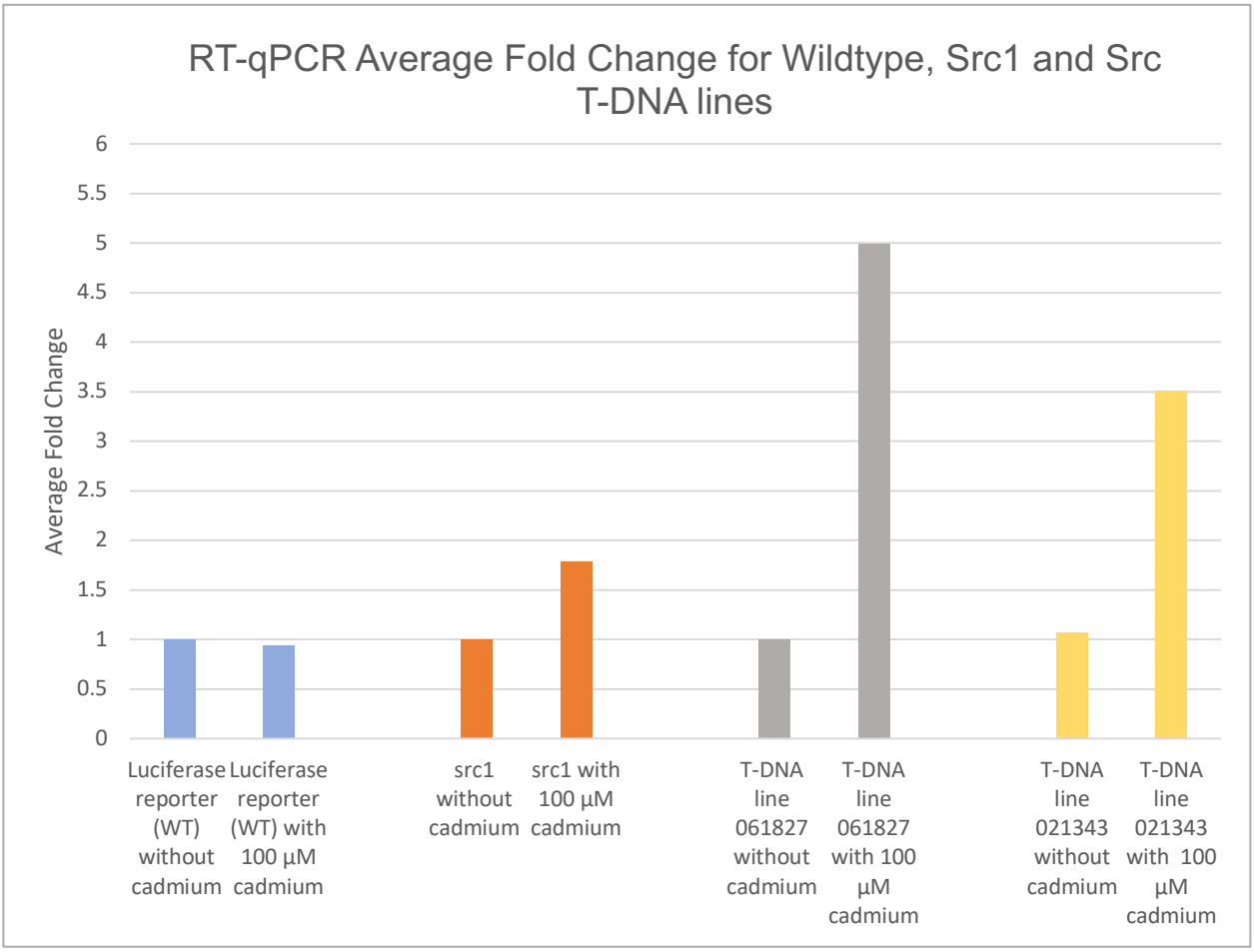

Figure 9: To quantify the average fold change in gene expression, Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) was conducted for "luciferase reporter line" (WT), mutant "super response to cadmium (src1)", SRC T-DNA insertion SALK_ line 061827 and 021343 (pseudogene *AT4G16267, and gene AT4G16270*). All lines were plated and grown on ¼ MS media for approximately 7 days. The plant seedlings were then taken to either control ¼ MS media plates with no cadmium or ¼ MS media with 100 µM of cadmium for approximately 6 hours. Data analysis using Delta-Delta Ct calculations was used. The average fold change values included 3 biological samples for all lines, except for the 061827 control gene of interest, with 2 biological samples due to technical issues.

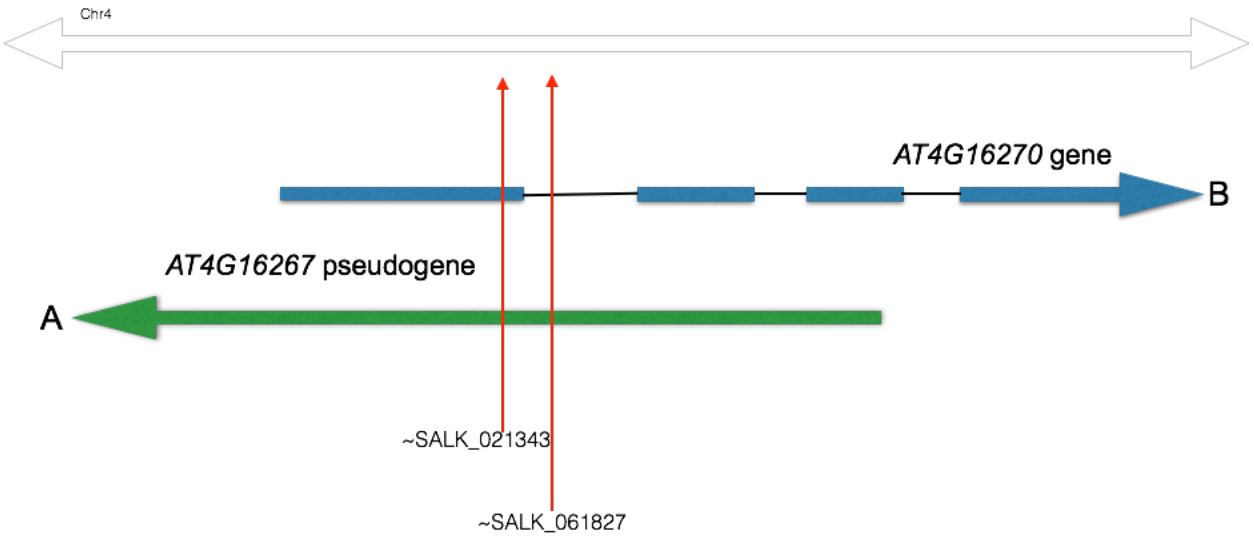

Figure 10: Visual approximation representation of overlapping gene and pseudogene A) Pseudogene *AT4G16267* B) Gene *AT4G16270*. Information for visual approximation gathered from TAIR (https://www.arabidopsis.org/) and Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu).

Discussion

Previously, “luciferase reporter lines” were made by merging the promoter region of cadmium-induced *SULTR1;2* gene with the bioluminescent firefly luciferase gene and transformation of *Arabidopsis thaliana* (Jobe et al., 2012). “Luciferase reporter lines” subjected to ethyl methanesulfonate (EMS) induced mutations (Jobe et al., 2012). Forward genetic screening of mutant lines displayed a phenotypic response in the presence of cadmium (Cd) exposure (Jobe et al., 2012). Three subgroups were defined as “constitutive response to cadmium (*crc1*)”, “super response to cadmium (*src1)”,* and “non-response & reduced response to cadmium (*nrc1,2*)” (Jobe et al., 2012). Bulk segregation analysis found 14 *crc1* and 25 *scr1* candidate mutations on chromosome 4 within the *Arabidopsis thaliana* genome (Cooper, 2018). T-DNA insertion knockout lines for mutant *crc1* and *src1* candidate genes were ordered to experimentally identify causative mutations (Cooper, 2018).

1.1 Mutant “Constitutive Response to Cadmium (*crc1*)” (Jobe et al., 2012)

Upon starting this project, several *crc1* lines exhibited substandard growth and seed germination. This finding could possibly be thought to be due to the age of the seeds. Therefore, propagation of *crc1* lines was pursued, and seeds that had adequate germination were obtained. Luciferase assays were conducted with the intent and purpose of confirming the existence of a functional luciferase reporter. Luciferase assay response experiments were conducted on the “luciferase reporter line” (WT), mutant “constitutive response to cadmium (*crc1*)”, and mutant “super response to cadmium (*src1*)” lines (Jobe et al., 2012). Luciferase assays displayed a weak luciferase luminescence for all lines (Figure 1). Possibly gene silencing could have taken place through the experimentation and propagation of lines for the luciferase reporter. Obtaining original seed stock for the purpose of propagation in order to conduct further luciferase assays may revive the weakly observed luciferase luminescence phenotypes.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was utilized to determine whether these mutants have distinct cadmium accumulation phenotypes compared to the luciferase reporter (WT) (Figure 2, Figure 3). All lines, when compared to themselves at the non-cadmium condition, 100μM of cadmium for 6 hours and 100μM of cadmium for 72 hours showed an increase in cadmium accumulation, respectively (Figure 2, Figure 3). These findings indicate that an increase in the exposure time to cadmium allowed for more accumulation for all three of the investigated lines. Compared to one another, all lines were found to have similar cadmium accumulation in the non-cadmium, 100μM of cadmium for 6 hours, and 100μM of cadmium for 72 hours (Figure 2, Figure 3). The preceding results provided the first evidence that no difference existed in cadmium accumulation for all lines within the same conditions.

Further repeat experiments with additional plates for each line can be conducted to investigate whether observable cadmium accumulation differences might exist. To determine if and to which extent cadmium accumulation was localized to the roots or shoots of the seedlings, further experiments conducting ICP-OES analysis are recommended with the separation of roots from the shoots after exposure to defined cadmium concentrations and exposure times. Furthermore, Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) can be conducted to investigate the gene expression for the luciferase reporter (WT), mutant *crc1, and src1* lines with and without the presence of cadmium, utilizing varying cadmium concentrations and durations. Additionally, ICP-OES analysis can be conducted to compare cadmium accumulation of the CRC and SRC candidate T-DNA insertion lines compared to the original *crc1* and *src1* mutants.

Previously, mutant *crc1* in 75μM of cadmium with 1μM of selenium exhibited an extended root length in sulfur-free media compared to the luciferase reporter (WT), as observed in the dissertation of Andrew Cooper (Cooper, 2018). Notably, the previously observed extended root growth for mutant *crc1* was no longer observed when compared to the luciferase reporter (WT) in the present study (Figure 5). The difference in root growth between genotypes

was within one standard deviation so that no firm conclusion can be drawn. Possibly poor seed quality could have taken place through the experimentation and propagation of the mutant *crc1* lines. Heeding the substandard growth and germination observed while starting this project for mutant *crc1* lines, poor seed quality is a feasible theory. To further evaluate these findings, root growth assays could be conducted at interval concentrations between 55-75μM of cadmium with and without 1μM of selenium with the original seed stock for mutant *crc1* lines.

For all genotypes within 100μM cadmium, inhibition of the average root length was noted and suggested that plant growth and survival are affected at this concentration (Figure 6). The difference in root growth between genotypes was within one standard deviation so that no firm conclusion can be drawn for all root length assays. CRC T-DNA insertion line 067394 (gene *AT4G10930)* was observed to have an extended average root length in 100μM cadmium with 1μM selenium when compared to cadmium without selenium (Figure 6). The difference in root growth between genotypes was within one standard deviation so that no firm conclusion can be drawn. These findings suggested that at higher concentrations of cadmium, the presence of selenium had the possibility of increased cadmium tolerance for CRC T-DNA insertion line 067394. CRC SALK_T-DNA insertion line 067394 (gene *AT4G10930)* displayed poor growth for all three-cadmium concentrations suggesting either poor seed quality or the gene is required for normal seedling development and growth, even in control experimental conditions. (Figure 4, Figure 5, Figure 6). Based on the observed data, root growth assays provided no apparent statistical differential effect between the *crc1* mutant and the parent controls. Further RT-qPCR experiments can be conducted to examine the gene expression of cadmium-induced *SULTR1;2* genes in CRC SALK_T-DNA insertion lines 117073 (gene *AT4G13575*), 120184 (gene *AT4G10930*), and 067394 (gene *AT4G10930*) compared to that of mutant c*rc1*.

The gene *AT4G10930*, according to The Arabidopsis Information Resource (TAIR) directory, is transcribed and translated to a RING/U-box protein and may also be associated

with the function of ubiquitination (https://www.arabidopsis.org/, Lamesch et al., 2012). An in-depth investigation of a ubiquitin ligase gene was identified to have the possibility of being associated with heavy metal tolerance (Qi et al., 2020). Adaptive strategies in unfavorable conditions need to be considered when examining the possible function of this gene. The gene *AT4G13575*, according to the TAIR directory, is transcribed and translated to a hypothetical protein (https://www.arabidopsis.org/, Lamesch et al., 2012). The gene *AT4G13575* was a gene of interest within *Arabidopsis* in the in-depth investigation regarding areas of chloroplast translation (Bailey et al., 2021). Plant's adaptive mechanisms for essential survival and development need to be considered when examining the possible function of this gene.

Future research will focus on finding gene mutations with an increased responsive cadmium-induced *SULTR1;2* gene expression as those previously reported for the observable mutant *crc1* phenotype. It needs to be considered that other candidate genes in the *crc1* rough mapping region not mentioned above could have the potential to represent the causative mutated gene in the *crc1* mutant. Further examination through research of candidate plant genes to investigate their involvement in the cadmium-induced gene expression response is needed.

1.2 Mutant "Super Response to Cadmium (*src1*)" (Jobe et al., 2012)

Previously RT-PCR discovered SRC T-DNA insertion genes *AT4G16267* and *AT4G15230* with increased SULTR1;2 gene expression within cadmium similar to mutant *src1* exhibited by higher intensity bands when compared to luciferase reporter (WT) (Scavo, 2019). As this previous research was conducted using the less quantitative RT-PCR approach, I pursued research to further quantitatively analyze candidate mutants. To assess candidate mutants for *src1* phenotype, Reverse Transcriptional quantitative Polymerase Chain Reaction (RT-qPCR) was conducted to assay cadmium-induced *SULTR1;2* gene expressions in

luciferase reporter (WT), mutant *src1*, and SRC SALK_T-DNA lines 061827 and 021343 (pseudogene *AT4G16267, and gene AT4G16270*) (Figure 9).

Quantitative analysis of gene expression using RT-qPCR revealed that SRC SALK_ T-DNA insertion lines 061827 and 021343 (pseudogene *AT4G16267, and gene AT4G16270)* both displayed increased *SULTR1;2* gene expression within cadmium, similar to or stronger than the mutant *src1* when compared to the luciferase reporter parent line (WT) (Figure 9). These findings are very promising because the data results suggested that mutations in these candidate genes could be responsible for or play a part in the mutant *src1* phenotype due to having a super transcriptional response within cadmium similar to mutant *src1*. Replicate RT-qPCR experiment, and future rescue experiments can be conducted to examine the gene expression of cadmium-induced *SULTR1;2* in SRC SALK_T-DNA lines 061827 and 021343 (pseudogene *AT4G16267, and gene AT4G16270*) compared to that of mutant *src1*. Rescue experiments would involve cloning the wild-type version of pseudogene *AT4G16267* and gene *AT4G16270* and expressing *src1* mutant in the background. If *src1* has a mutation in one or both genes, the wild-type gene of interest will rescue the phenotype, and the super response in the presence of cadmium would no longer be observed. After checking for successful transformation, RT-qPCR or luciferase assay experiments can be conducted to find the possible genes responsible for the mutation in the *src1* phenotype.

Interestingly, pseudogene *AT4G16267* and gene *AT4G16270* are on opposite strands and have overlapping regions (Figure 10) (https://www.arabidopsis.org/, http://signal.salk.edu). For this reason, CRISPR-CAS9 gene editing can be used to target the separate regions on separate strands to repair the genome insertion and conduct experiments to see if the mutant *src1* phenotype is still observed. The gene *AT4G16270*, according to the TAIR directory, is transcribed and translated to class III peroxidase (https://www.arabidopsis.org/, Lamesch et al., 2012). Two separate class III peroxidase genes had an in-depth investigation and became the research focus regarding specific areas pertaining to development and structure

within *Arabidopsis* (Jacobowitz et al., 2019). The heavy metal response to cell wall structure needs to be considered when examining the structure-function aspect of this gene.

The pseudogene *AT4G16267*, according to the TAIR directory, is transcribed and translated to a plant thionin family (https://www.arabidopsis.org/, Lamesch et al., 2012). Plant thionins have been involved in several defense responses, including pathogens (Bohlmann & Broekaert,1994, Ali et al., 2018). Recognized pathogen invasion is an important aspect to be considered when examining the function of a plant thionin. The environmental cues that signal response, such as the heavy metal response, may need to be examined when considering a plant thionin gene.

Future research will focus on finding gene mutations with an increased responsive cadmium-induced *SULTR1;2* gene expression as those previously reported for the observable mutant *src1* phenotype. It needs to be considered that other candidate genes in the *src1* rough-mapping locus, not mentioned above, may be responsible for the causative mutation presented in the *src1* phenotype. However, the presented RT-qPCR experiments provided initial evidence that one of the two overlapping genes analyzed here may function in cadmium-induced gene expression. Therefore, further RT-qPCR repeat experiments will be necessary.

Material and Methods

1.1 Protocol for Lab Attire

Lab coat, safety goggles/safety glasses, safety latex gloves, and safety heat resistant gloves were worn for safety during appropriate sections for experiments. Safety and sterile hoods were utilized during proper sections of experiments.

1.2 Protocol for Seed Sterilization

A concentration of 33% bleach solution with Germicidal ultra-bleach was used to sterilize seeds (Scavo, 2019). Approximately 500 µM of bleach solution was distributed to each 1.5mL Eppendorf sterile tube with seeds inside. Seeds were determined by design for specific experiments. All seeds were submerged in the bleach solution and given a vigorous shake for approximately 45 seconds. The Eppendorf tubes were stationary for approximately 5 minutes under the sterile hood with the bleach solution inside. The bleach solution was then removed using a 1000 µl pipette tip. Approximately 500 µM of sterilized water was added to every tube with seeds inside. Each tube was given a vigorous shake for approximately 45 seconds. The water inside the microcentrifuge tubes was removed, and new sterile water was added. The removal and addition of fresh sterile water were done for approximately five rotations. The seeds were then placed at approximately 4˚C for 72 hours. Seeds were then placed on media appropriate for the experiment underneath a sterile hood, and plates were sealed using micropore tape. The taped plates were placed vertically unless otherwise specified into the growth chamber room set to 16-hours of light and 8- hours of dark rotation at approximately 21 ˚C.

1.3 Protocol for Plant Growth

Plants had been grown using Sungro's professional growing mix. The soil was sterilized through autoclave before use. The dry application of approximately 1 tbsp of Mighty Myco water-soluble

premium mycorrhizal & bacterial inoculant was added for approximately every 8 gallons of sterile soil. Following sterilization and cold treatment, *Arabidopsis thaliana* seeds were plated and grown on sulfur-free media and then taken to pots filled with soil after approximately 1-2 weeks. Plants were placed into the growth chamber room set to have 16-hours of light and 8-hours of dark rotation at a temperature of approximately 21˚C. Transparent plastic hoods were placed over the trays, with the ventilation holes found at the top of the transparent plastic hood turned to the fully opened position. The transparent plastic hoods remained over the newly potted plants for approximately 5-6 days and were then removed.

1.4 Genotypes Used

Mutant lines used for experiments included “super response to cadmium (*src1*)” and “constitutive response to cadmium (*crc1*)” (Jobe et al., 2012). “Luciferase reporter lines” (316/WT) were used as a control/wild-type in the experiments (Jobe et al., 2012). SRC T-DNA insertion candidate lines used included SALK_021343 and SALK_061827 (pseudogene *AT4G16267* and gene *AT4G16270*). CRC T-DNA insertion candidate lines used included, SALK_067394, SALK_120184 (gene *AT4G10930*) and SALK_117073 (gene *AT4G13575*). If cadmium is mentioned in the thesis, it refers to $CdCl_2$. Andrew Cooper provided the information for *src1* and *crc1* candidate gene mutations and the primer list in the laboratory of Julian Schroeder and can be found in the dissertation of Andrew Cooper (Cooper, 2018). SALK insertion lines: Arabidopsis Biological Resource Center (ABRC); (https://abrc.osu.edu/, Alonso et al., 2003, http://arabidopsis.org/abrc/, http://signal.salk.edu).

1.5 Protocol for Pouring Plates

Approximately 25mL of the desired media for the experiment with the use of an Accu-jet pro was dispensed onto a sterile polystyrene petri dish with grids pre-marked on the plate. Plates were poured underneath a sterile hood.

1.6 Protocol for Sulfur Free Media Concentration

Sulfur-free media consisted of the final concentrations for approximately 1L of solution: .25mM of Ca (No3)2, .001mM of CuCl2, .03mM of H3BO3, .05mM of KCl, 1.0mM of KH2PO4, .5mM of KNO3, 1mM of MgCl2, .01mM of MnCl2, .1mM of Na (EDTA)Fe, .0001mM of (Nh4)6 (Mo7O2)4, .001mM of ZnCl2 (Cooper, 2018, Scavo, 2019). The pH was modified to approximately 5.6 utilizing KOH. Tissue culture grade Phyto agar was added to reach approximately 1.5% agar. The approximately 1L solution of sulfur-free media was then poured into two 1000mL bottles evenly (approximately 500mL of sulfur-free media solution in each bottle) and sterilized.

1.7 Protocol for ¼ MS with Sucrose Media Concentration

¼ MS with sucrose media consisted of approximately 1L of solution: approximately 1.1g Murashige and Skoog (MS), approximately 0.2g MES hydrate, and approximately 1% Sucrose (Cooper, 2018, Scavo, 2019). The pH was modified to approximately 5.6 utilizing KOH. Tissue culture grade Phyto agar was added to reach approximately 1.5% agar. The approximately 1L solution of ¼ MS with sucrose media was then poured into two 1000mL bottles evenly (approximately 500mL of ¼ MS with sucrose media solution in each bottle) and sterilized.

1.8 Protocol for ¼ MS Media Concentration

¼ MS media consisted of approximately 1L of solution: approximately 1.1g Murashige and Skoog (MS), and approximately 0.2g MES hydrate (Cooper, 2018, Scavo, 2019). The pH was modified to approximately 5.6 utilizing KOH. Tissue culture grade Phyto agar was added to reach approximately 1.5% agar. The approximately 1L solution of ¼ MS media was then poured into two 1000mL bottles evenly (approximately 500mL of ¼ MS media solution in each bottle) and sterilized.

1.9 Protocol for Root Growth Assays

For root growth assays, plant seeds were plated and grown on sulfur-free media plates and grown for approximately 7 days. Plant seedlings were then taken using forceps under a sterile hood to either control sulfur-free media plates or treatment (containing cadmium ($CdCl_2$) with and without selenium) sulfur-free media plates for another approximately 7 days of exposure growth and then photographed. The day the plant seedlings were taken to new plates, a black sharpie dot was placed on the back of the new plates at the end of the primary seedling root to indicate the initial root length. Three replicate plates for each treatment were measured using Image J software.

1.10 Protocol for Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

Plant seedlings were plated and grown for approximately 14 days on sulfur-free media plates for ICP-OES. The plant seedlings were then collected for control or taken onto treatment plates containing sulfur-free media with 100 µM of cadmium ($CdCl_2$) for approximately 6 hours and 100 µM of cadmium ($CdCl_2$) for approximately 72 hours. Plates were laid vertically for growth and exposure time. Three replicate tray samples were collected for all lines. The wet weights and dry weights were recorded. Nitric acid (70% purified by redistillation) was added to samples, and samples were placed in a boiling water bath underneath the fume hood. Safety goggles were worn, and the protective shield for the fume hood was lowered to a safe level. Samples were centrifuged for approximately 20-25 minutes. 1 mL of the supernatant was taken to sterile 15 mL centrifuge tubes, with 2 mL of sterile water added. Tubes were covered with parafilm tape and sent for analysis at the Danforth Center for ICP-OES. The following formula was used for data analysis (ICP-OES PPB X 0.003)/dry weight in grams.

1.11 Protocol for Luciferase Assays

Plant seedlings were plated and grown on ¼ MS with sucrose media plates for luciferase assays. Nylon mesh of 80 microns was cut to fit inside the plates and sterilized. Plant seeds were plated and grown on ¼ MS with sucrose media overlaid with sterile 80-micron nylon mesh to facilitate the bulk seedling transfer. After approximately 7 days, the plant seedlings on nylon mesh were then taken to ¼ MS with sucrose plates that had approximately 100uL of 100 µM of luciferin distributed over the entirety of the plates on the 8th day and were set to sit for 24 hours. The plates were covered with foil to ensure limited light penetration and laid horizontal in the growth chamber. The plant seedlings were then consequently taken to either ¼ MS with sucrose plates or treatment ¼ MS with sucrose plates with 100 µM of cadmium. Both of the plates had approximately 100uL of approximately 100 µM of luciferin distributed on the media. The distribution of luciferin was done under a dark and sterile hood in a dark room. All plates were covered with foil once luciferin was distributed to ensure minimal light penetration. Plates were laid horizontally in the growth chamber room for approximately 6 hours before imaging with the Berthold Night Owl imaging system (Cooper, 2018, Scavo, 2019).

1.12 Protocol for Reverse Transcription- Quantitative Polymerase Chain Reaction (RT-qPCR)

Plant seedlings were plated and grown on ¼ MS media plates for RT-qPCR for approximately 7 days. The plant seedlings were then taken to either control ¼ MS media plates with no cadmium or ¼ MS media with 100 µM of cadmium for approximately 6 hours. After approximately 6 hours, all plant seedlings were collected and frozen in liquid nitrogen with 2.3mm metal beads inside the tubes. The plant tissues were ground for three minutes using a Mixer Mill. Total RNA was isolated using a Sigma-Aldrich Spectrum Plant Total RNA Kit consistent with the manufacturer's protocol. Consistent with the manufacturer's protocol, RNA samples were treated with DNase from the Turbo DNA- free KIT Invitrogen by Thermo Fisher Scientific. RNA sample concentrations were measured using a nanodrop spectrophotometer. The First-strand cDNA synthesis kit by GE healthcare was carried out consistent with the manufacturer's protocol.

cDNA sample concentrations were measured using a nanodrop spectrophotometer. A C1000 Thermal Cycler was used to perform a thermal gradient block PCR to establish the ideal annealing temperature. The following temperatures for the following steps were used for PCR thermal gradients and qPCR: 94.0 ˚C denature, 65 ˚C annealing, and 72 ˚C DNA synthesis with 40 cycles using Bio-Rad CFX machine. Wells for qPCR contained approximately 1 µL of primer *AT1G78000.1* (SULTR1;2) or *AT5G60390* (EF1α housekeeping gene), approximately 10 µL Master Mix containing Taq polymerase, dNTPs and SYBR Green, approximately 5 µL of sterile DI water, and approximately 3 µL of cDNA of the desired line. The calculation of delta delta ct was used for data analysis.

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