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Analysis of Heavy Metal Phytostabilization Approach in Oryza sativa (Rice) with Root-targeted Overexpression of AtHMA3 and TaPCS1

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Analysis of Heavy Metal Phytostabilization Approach in *Oryza sativa* (Rice) with Root-targeted Overexpression of *AtHMA3* and *TaPCS1*

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Dikran Khachadourian

Committee in charge:

> Professor Julian I. Schroeder, Chair
> Professor Alisa Huffaker, Co-Chair
> Professor Jose Pruneda-Paz

2021

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

University of California San Diego

2021

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I would like to thank my family for all their unconditional love and sacrifices. I would not have been able to explore such amazing opportunities and be where I am without your constant support through everything. My appreciation for all that you have done for me is beyond anything words can express.

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Figure 1 is coauthored with Khachadourian, Dikran and Yu, Qi. The thesis author was a coauthor of this figure.

ABSTRACT OF THE THESIS

Analysis of Heavy Metal Phytostabilization Approach in *Oryza sativa* (Rice) with Root-targeted Overexpression of *AtHMA3* and *TaPCS1*

by

Dikran Khachadourian

Master of Science in Biology

University of California San Diego, 2021

Professor Julian I. Schroeder, Chair

Professor Alisa Huffaker, Co-Chair

The industrial and urban evolution of the modern world has contributed to the innovative developments and economic growth of many countries. This growth has also resulted in elevated levels of heavy metal pollutants in the biological systems of these nations, such as their cropland and bodies of water. This becomes a predicament, as some of these nations are leading producers of agricultural products worldwide. Since the exposure of heavy metal toxicants *via* ingestion of contaminated crops can cause many serious illnesses to humans, this dilemma has received

global attention from health organizations and science communities. To contribute to these efforts, this thesis research project aims to evaluate a heavy metal phytoremediation technique in rice plants known as "phytostabilization". Phytostabilization refers to the immobilization of heavy metal pollutants in the roots of plants to prevent their uptake into the shoots, leaves, and seeds. This was tested with transgenic rice lines with root-targeted overexpression of the following transgenes: Phytochelatin Synthase Gene from *T. aestivum* (*TaPCS1)* and Heavy Metals Associate3 gene from *A. thaliana* (*AtHMA3*). *TaPCS1* overexpressing rice lines were exposed to different concentrations of both cadmium ($CdCl_2$) and arsenite (As(III)), while *AtHMA3* overexpressing lines were only exposed to different concentrations of cadmium ($CdCl_2$). ICP-MS analysis was utilized to measure heavy metal contents in the root vs. shoot tissues of the aforementioned rice lines upon exposure to their respective heavy metal conditions. The preliminary results of this study suggested that rice lines with root-targeted overexpression of *AtHMA3* were able to achieve lower Cd accumulation in the shoot tissues when compared to the wildtype controls. Furthermore, there was promising evidence that rice lines with root-targeted overexpression of *TaPCS1* exhibited lower Cd and As(III) accumulation in the shoot tissues than the wildtype controls; however, further experimentation must be done on multiple independent transgenic lines and several exposure regimens.

Introduction

  Pollution takes many forms and can cause a wide variety of disturbances to our planet's

biological systems and organisms. Heavy metal contamination is one form of pollution that has

been a global dilemma due to the adverse effects it has on agriculture and human health. The

term "heavy metal" refers to the number of metal/metalloids with relatively high atomic weights

and densities (Tchounwou et al., 2012). Due to these chemical properties, heavy metals are

known to have toxic effects on plants, animals, and humans, as exposure to these toxicants may

result in the hindrance of natural biological processes (Li et al., 2019). In addition to the toxic

effects they may have on organisms, heavy metals are non-degradable, making their presence as

irreversible contaminants a difficult and complex issue to address (Zwolak et al., 2019;

Paithankar et al., 2021; Zhang et al., 2021).

  Heavy metal contamination can occur due to both natural and anthropogenic occurrences.

The earth's crust is one source of heavy metal and metalloid contaminants where they can

surface through natural processes, such as erosion and surface winds (Khatri., 2015; Li et al.,

2019) . The majority of heavy metal pollution, however, can be attributed to anthropogenic

sources, such as mining/smelting, the use of pesticides, and improper disposal of hazardous

waste (Alkorta et al., 2007; Bolan et al., 2011; Zhang et al., 2021). Through these activities,

heavy metals such as cadmium and arsenic can make their way into soils where crops are

cultivated. Upon consumption of water and nutrients from the contaminated soil in which they

are grown, crops can manage to take up and accumulate these non-essential heavy metal

contaminants in the process. Contaminated plants may experience toxicity and oxidative stress,

as heavy metals have been shown to promote the production of reactive oxygen species in plants

(Clemens, 2006; Song et al., 2014; Chaudhary et al., 2016). This also allows for the entry of

toxic heavy metals and metalloids into the food chain, resulting in further consumption by animals and humans. Moreover, such heavy metal exposure may lead to a multitude of diseases and complications in humans. Cadmium-related diseases may include different types of cancers, hypertension, and peripheral artery disease (Mendoza-Cózatl et al., 2010; Li et al., 2019; Cooper et al., 2020). Arsenic exposure can also cause complications to the human body, such as neurological effects, diseases of the respiratory system, and different forms of skin lesions (Lee et al., 2003; Li et al., 2019).

The rapid rates of industrialization and urbanization in the preceding decades have resulted in many global advancements. Unfortunately, these advancements came at the cost of increased pollution in our planet's atmosphere, bodies of water, and agricultural land (Li et al., 2019). Countries located in Asia, such as China and India, have some of the highest industrial outputs; thus, these nations have experienced a drastic increase in harmful toxins in their biological systems (Lee et al., 2003; Sharma et al., 2007; Wu et al., 2015; Li et al., 2019) Cropland is one system to fall victim to such contamination, as the presence of heavy metals/metalloids such as cadmium and arsenic have been detected in agricultural rice paddies and crops. This is a dilemma as Asia is a leading rice supplier and is responsible for ~90% of the rice production around the world (Bandumula et al.. 2018). Since rice is a valuable staple crop for many civilizations worldwide, the concern of heavy metal contamination and accumulation of rice crops has become a heavily studied topic. In Japan, studies have previously shown the emergence of *itai-itai* disease amongst a portion of the population due to cadmium exposure *via* contaminated rice crops (Nogawa et al., 1983; Arao et al., 2010; Chaney., 2015). Such occurrences have led to the involvement of science communities and health organizations around

the world, such as the World Health Organization, in a collaborative effort to closely monitor

and minimize the effects of heavy metal exposure (Järup, 2003).

Scientific researchers contributing to these global efforts have taken the approach of

remediation to address the issue of heavy metal and metalloid contamination of crops. Different

forms of remediation have been researched, including physical remediation, chemical

remediation, bioremediation, and phytoremediation (Li et al., 2019). Physical remediation

includes processes through which contaminated soil is completely replaced or isolated from

clean soil. This approach is costly and inefficient, making it difficult to implement to larger

scales. Chemical remediation entails the use of chemical reagents to remove heavy metal

contaminants from the soil (Arao et al., 2010). An example of this is washing contaminated soil

with ethylenediaminetetraacetic acid (EDTA), a chelating agent (Li et al., 2019). Although

effective, this approach is still high in cost and can lead to secondary contaminants, as well as

runoff of metals into groundwaters. One of the more modern approaches is bioremediation, a

process through which the inherent properties of microorganisms, such as fungi and bacteria, are

utilized to remove or immobilize heavy metals in contaminated soil (Li et al., 2019). Although

effective, this approach is hard to implement in situations where contaminants cover large areas

and/or where heavy metals have already been exposed to the roots of cultivated crops. Falling

under the umbrella of bioremediation, phytoremediation implements plant species to immobilize

or reduce the toxicity of heavy metals upon exposure (Chaudhary et al., 2016; Li et al., 2019).

Phytoremediation is flexible and effective, as multiple biological techniques and processes can

be utilized to achieve optimal results.

This project aims to implement a phytoremediation technique known as

"phytostabilization" to expand on the ongoing research regarding heavy metal contamination of

rice plants. Phytostabilization is a process through which plants can immobilize toxic heavy metals and metalloids in the soil, preventing further absorbance into the shoots and edible portions of the plant (Bolan et al., 2011). This can be done by overexpressing specific genes in the roots of rice plants to sequester the heavy metal/metalloid(s) of interest in the root tissues. When overexpressed, these genes have been shown to detoxify heavy metals through their respective mechanisms. One such gene that was previously discovered in (wheat) is phytochelatin synthase gene (PCS1), which plays a role in the detoxification of cadmium and arsenic through the production of phytochelatins (Chen et al., 2006; Mendoza-Cózatl et al., 2011). Phytochelatins (PC) form complexes with these heavy metals and metalloids to ultimately reduce their toxicity. PC-Cd and PC-As(III) complexes are then sequestered into the vacuole through an ATP-Binding Cassette transporter (Abc2/ABCC2) (Mendoza-Cózatl et al., 2011). Belonging to the P-type ATPase protein family, Heavy Metal Associated3 (HMA3) is another gene that has previously been discovered to play a similar role in cadmium detoxification (Mendoza-Cózatl et al., 2011; Liu et al., 2017). As a tonoplast-localized cadmium pump, HMA3 is able to detoxify cadmium by transporting and sequestering these contaminants in plant vacuoles (Mendoza-Cózatl et al., 2011).

In order to utilize these genes to test the technique of phytostabilization of cadmium and arsenic in rice plants, transgenic rice lines were prepared with the transgenes PCS1 and HMA3. These lines, however, were transformed with vectors containing a root-specific promoter rather than a full-plant promoter. This root-specific promoter ensures that these transgenes are solely overexpressed in the roots of the rice plants to achieve cadmium and arsenic sequestration in the roots and prevent further localization in the different tissues of the plant.

Within the presented Master thesis research, transgenic rice plants overexpressing TaPCS1 and AtHMA3 genes under the control of the rice root-specific promoter were grown, seeds were harvested and exposed to different heavy metal conditions. Heavy metal contents in the root versus shoot tissues of treated rice plants were then measured using Inductively Coupled Plasma- Mass Spectrometry (ICP-MS). The present study provides preliminary evidence that root-targeted expression of selected transgenes could aid in avoiding arsenic and cadmium accumulation in leaves, which could provide an approach for reduced accumulation in seeds.

Results

ICP-MS Analysis

Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis was performed on successfully transformed rice lines overexpressing *Arabidopsis thaliana* Heavy Metal Associated3 (AtHMA3) and *Triticum aestivum* phytochelatin synthase (TaPCS1) in their roots. Polymerase Chain Reaction (PCR) genotyping was initially conducted to confirm that these constructs were present in investigated rice lines (Figure 1). **Figure 1-A** shows the presence of the PCS1 transgene in TaPCS1 rice lines and the absence of PCS1 in the wildtype controls. **Figure 1-B** shows the presence of the HMA3 transgene in the AtHMA3 rice lines and the absence of HMA3 in the wildtype controls. **Table 1** presents the primer pairs designed for these PCR genotyping experiments. ICP-MS is used to measure heavy metal and metalloid accumulation in plant tissues. The objective of this analysis is to observe the difference in heavy metal/metalloid accumulation in root/shoot tissues of root-targeted transgenic TaPCS1 and AtHMA3 rice lines when compared to the Nipponbare wildtype controls. Observing a difference in heavy metal/metalloid accumulation in the roots versus the shoots of these transgenic lines will further contribute to the understanding of phytostabilization, a process through which heavy metals become trapped in the root tissues of the plants.

1.1 ICP-MS Analysis of AtHMA3

AtHMA3 transgenic rice plant lines (HMA3-1/HMA3-2) were first exposed to 55µM of $CdCl_2$ for 72 hours. ICP-MS analysis showed that there was lower cadmium accumulation in the shoot tissues of HMA3-1 when compared to the shoot tissues of the wildtype controls. However, there was higher cadmium accumulation in the shoot tissues of HMA3-2 when compared to the shoot tissues of the wildtype controls. This difference in cadmium accumulation in the root tissues of HMA3-2 and the wildtype was consistent with that of the shoot tissues, as there was higher cadmium content in the HMA3-2 root tissues as well. There was no significant difference in the cadmium accumulation in the root tissues of HMA3-1 when compared to the root tissues of the wildtype controls (Figure 3).

To further investigate whether the difference in the cadmium accumulation in the shoot tissues of HMA3-1 when compared to the wildtype controls at 55μM $CdCl_2$ is of statistical significance, a T-test was used for statistical analyses. With a P-value of 0.0223, the T-test results suggested that there was a significant difference in the cadmium accumulation between the shoot tissues of HMA3-1 and shoot tissues of the wildtype controls (Figure 4). One-way ANOVA and Tukey's test was also performed to further confirm that there was a statistical difference in cadmium content in the shoot tissues of HMA3-1 when compared to control conditions.

Treatment conditions were altered for the second round of ICP-MS experiments by lowering the $CdCl_2$ concentration from 55μM to 2μM to further test previous findings. When comparing AtHMA3 lines to the wildtype, no significant difference in cadmium accumulation in the root and shoot tissues of the plants was observed (Figure 5).

1.2 ICP- MS Analysis of TaPCS1

In the first round of ICP-MS analysis, TaPCS1 transgenic rice plants (PCS1-1/PCS1-2) were treated with a mixture of 10μM As(III) and 50μM $CdCl_2$. A significant difference cannot be observed when comparing the cadmium accumulation in the root/shoot tissues of PCS-1/2 and wildtype controls (Figure 6). As 50μM of cadmium is a relatively high concentration, a second round of ICP-MS experimentation was performed with an altered concentration of $CdCl_2$ of 5μM, while keeping the concentration of As(III) at 10μM (Figure 7).

In these experiments of ICP analysis of PCS1-1/PCS1-2 suggested a significance in arsenite and cadmium accumulation was not observed when comparing the root tissues of the wildtype and transgenic lines. However, lower accumulation of both cadmium and arsenite accumulation can be observed in the shoot tissues of PCS1-2 when compared to the shoot tissues of the wildtype control (Figure 7). To further analyze the significance of this observable difference, a graph was generated with a focus on the shoot tissues of PCS1-2 and wildtype control when compared to control conditions where plants were not

exposed to any heavy metals. T-test statistical analysis showed that there was no significant difference in

arsenite and cadmium accumulation between PCS1-2 and wildtype shoot tissues (*P>0.05*) (Figure 8).
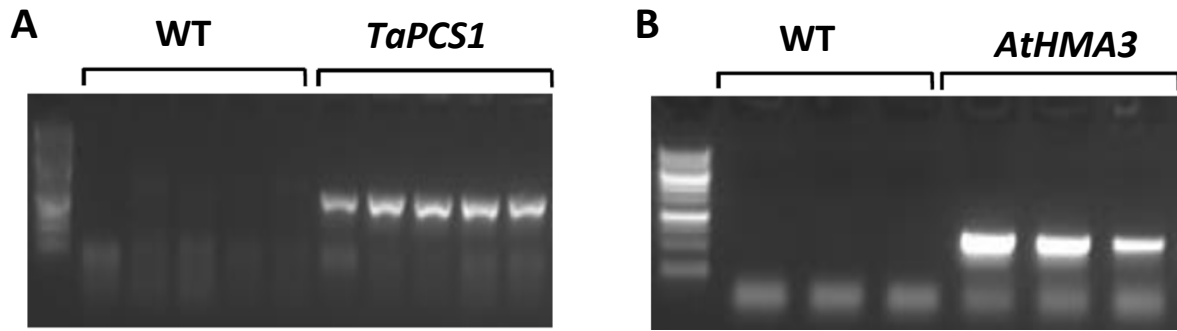
Figure 1: Polymerase Chain Reaction (PCR) genotyping data confirming transgenes in transformed rice lines. **A)** PCR data for Nipponbare wildtype and *Ta*PCS1 overexpressing lines. **B)** PCR data for Nipponbare wildtype and *At*HMA3 overexpressing lines. **Figure 1** is coauthored with Khachadourian, Dikran and Yu, Qi. The thesis author was a coauthor of this figure.

Table 1: List of primer pairs used for PCR genotyping experiments shown in Figure 1. Primer pairs were designed for transgenes TaPCS1 and AtHMA3.

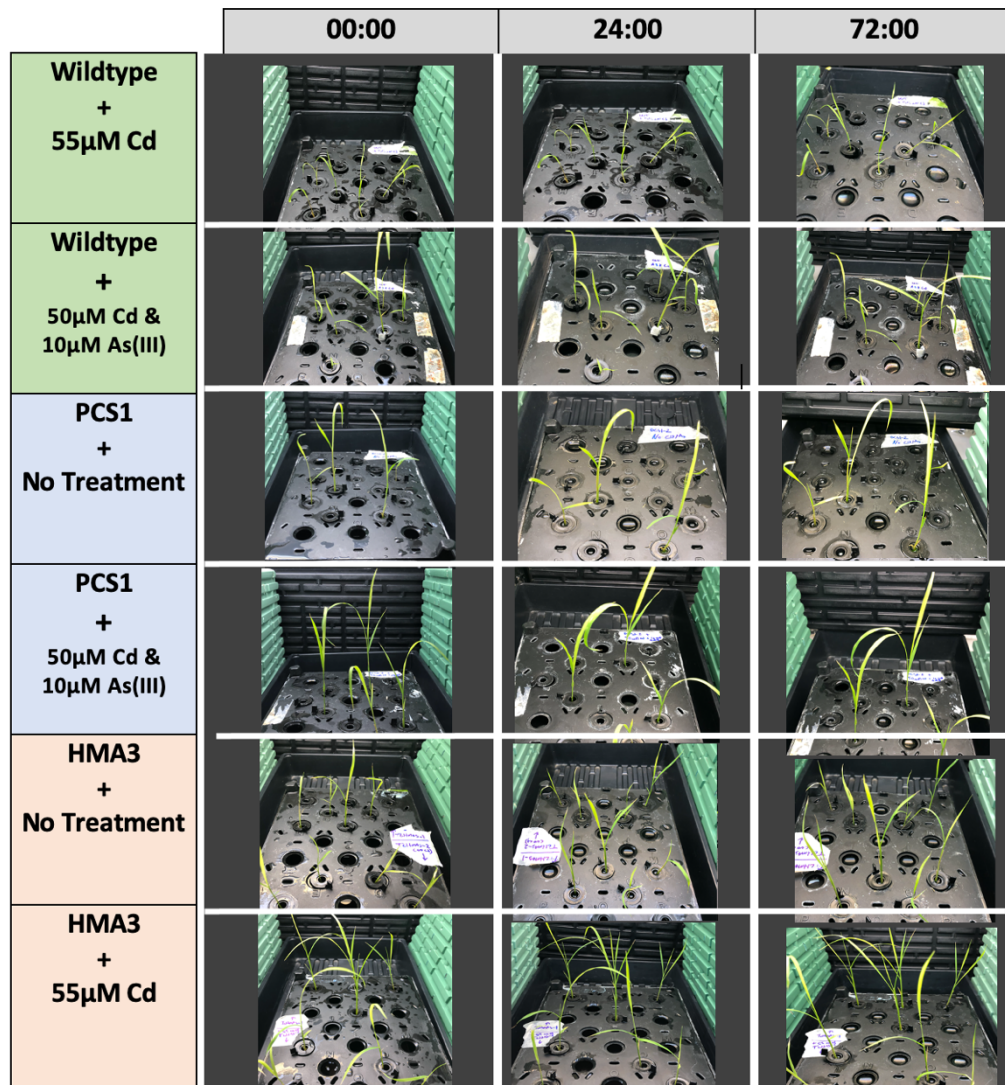| Gene of Interest	Primer Sequence
TaPCS1-Forward Primer	ATGGAGGTGGCGTCGCTG
TaPCS1-Reverse Primer	CTAAGGGGATGGAGGCTCTTGAC
AtHMA3- Forward Primer	ATGGCGGAAGGTGAAGAGTC
AtHMA3- Reverse Primer	TCATCCTTTCACTTCACCGGAGTTC

Figure 2: Images of Wildtype, AtHMA3, and TaPCS1 captured upon being exposed to their respective treatment conditions in hydroponic chambers at the following time intervals: 00:00 h, 24:00 h, and 72:00 h. These images were captured as a form of monitoring the plants for toxicity.
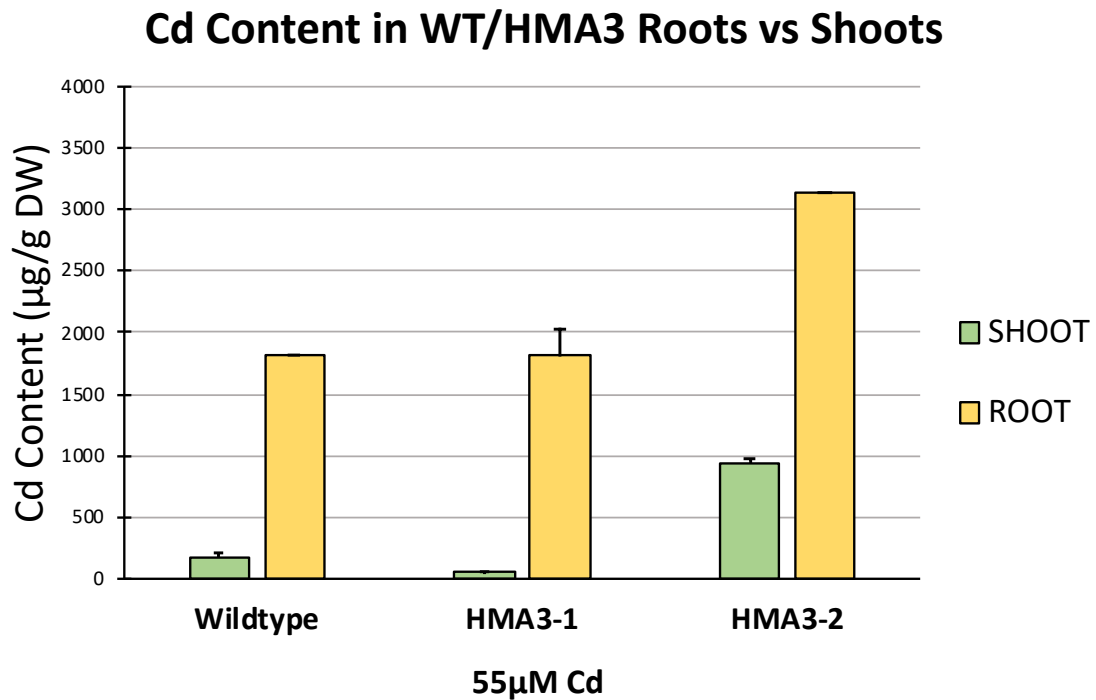
Cd Content in WT/HMA3 Roots vs Shoots

Figure 3: Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis measuring cadmium content in shoot vs. root tissues of Nipponbare wildtype and *Arabidopsis thaliana* Heavy Metal ATPase (AtHMA3) overexpressing rice (Oryza sativa) lines. Rice seeds were germinated on water-soaked paper towels in Petri dishes for 7 days. Upon germination, seedlings were transferred to hydroponic chambers and treated with hydroponic nutrients (P_2O_5, K_2O, MgO, SO_3, N, K_2O, CaO, Cu, Fe, Mn, Mo, Zn) for 21 days. On day 22, healthy wildtype and transgenic AtHMA3 rice plants were treated with 55μM of $CdCl_2$ for 72-hours, where they were monitored for toxicity. Plant materials were harvested before toxicity was clearly visible. Roots of treated plants were then washed with 5mM of $CaCl_2$. Shoots and Roots were separated and dried at 37° C for 7 days. Tissue samples were digested in 70% Nitric Acid for ICP-MS measurements. Error bars represent SEM (n=3).

Figure 4: Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis comparing cadmium content in shoot tissues of Nipponbare wildtype and *Arabidopsis thaliana* Heavy Metal ATPase (AtHMA3) rice lines. Control conditions were exposed to hydroponic nutrients only. Experimental conditions were treated with 55μM of CdCl$_2$ for 72-hours. Error bars represent SEM (n=3). **A)** T-test was used for statistical analyses (*P-Value = 0.0223*). **B**). One-way ANOVA and Tukey's test were used for statistical analyses.

Cd Content in WT/ HMA3 Roots vs Shoots

2µM Cd

Figure 5: Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis measuring cadmium content in shoot vs. root tissues of Nipponbare wildtype and *Arabidopsis thaliana* Heavy Metal ATPase (AtHMA3) overexpressing rice (Oryza sativa) lines. Rice seeds were germinated on water-soaked paper towels in Petri dishes for 7 days. Upon germination, seedlings were transferred to hydroponic chambers and treated with hydroponic nutrients (P_2O_5, K_2O, MgO, SO_3, N, K_2O, CaO, Cu, Fe, Mn, Mo, Zn) for 21 days. On day 22, healthy wildtype and transgenic AtHMA3 rice plants were treated with 2µM of $CdCl_2$ for 72-hours, where they were monitored for toxicity. Roots of treated plants were then washed with 5mM of $CaCl_2$. Shoots and Roots were separated and dried at 37° C for 7 days. Tissue samples were digested in 70% Nitric Acid for ICP-MS measurements. Error bars represent SEM (n=7 for WT; n=7 for HMA3-1; n=3 for HMA3-2).
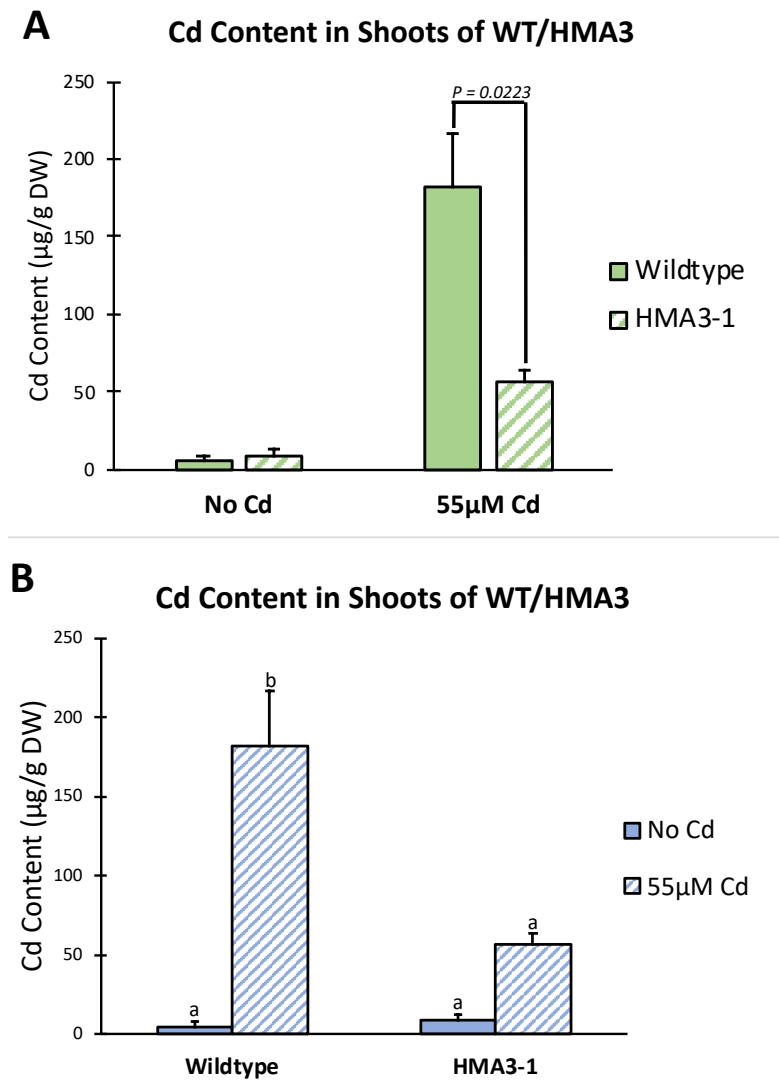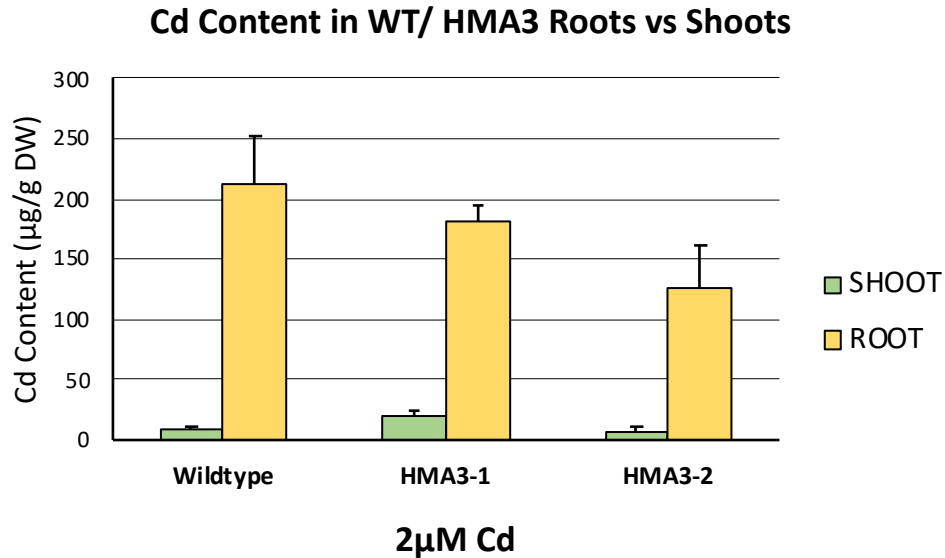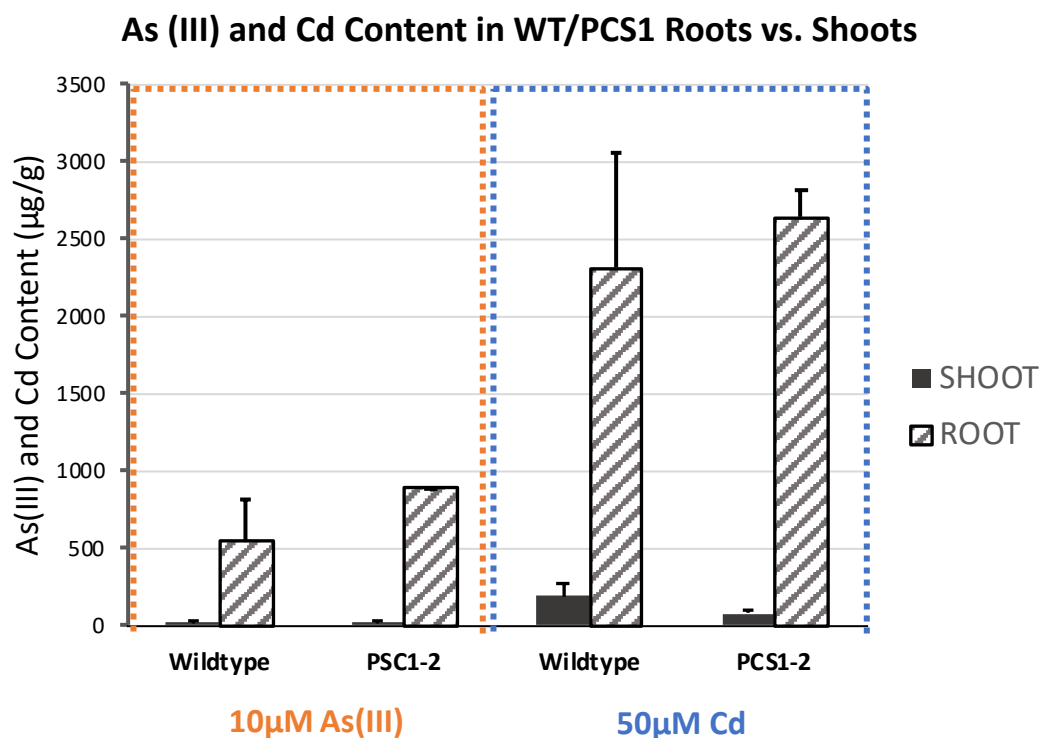
As (III) and Cd Content in WT/PCS1 Roots vs. Shoots

Figure 6: Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis measuring arsenite and cadmium content in shoot vs. root tissues of Nipponbare wildtype and *Triticum aestivum* phytochelatin synthase gene (TaPCS1) overexpressing rice lines. Rice seeds were germinated on water-soaked paper towels in Petri dishes for 7 days. Upon germination, seedlings were transferred to hydroponic chambers and treated with hydroponic nutrients (P_2O_5, K_2O, MgO, SO_3, N, K_2O, CaO, Cu, Fe, Mn, Mo, Zn) for 21 days. On day 22, healthy wildtype and transgenic TaPCS1 plants were treated with 10μM of As(III) and 50μM of $CdCl_2$ for 72-hours, where they were monitored for toxicity. Roots of treated plants were then washed with 1mM of K_2HPO_4 and 5mM of $CaCl_2$. Shoots and Roots were separated and dried at 37° C for 7 days. Tissue samples were digested in 70% Nitric Acid for ICP-MS measurements. Error bars represent SEM (n=3).

A

As (III) and Cd Content in <u>PCS1-1</u> Roots vs Shoots

B

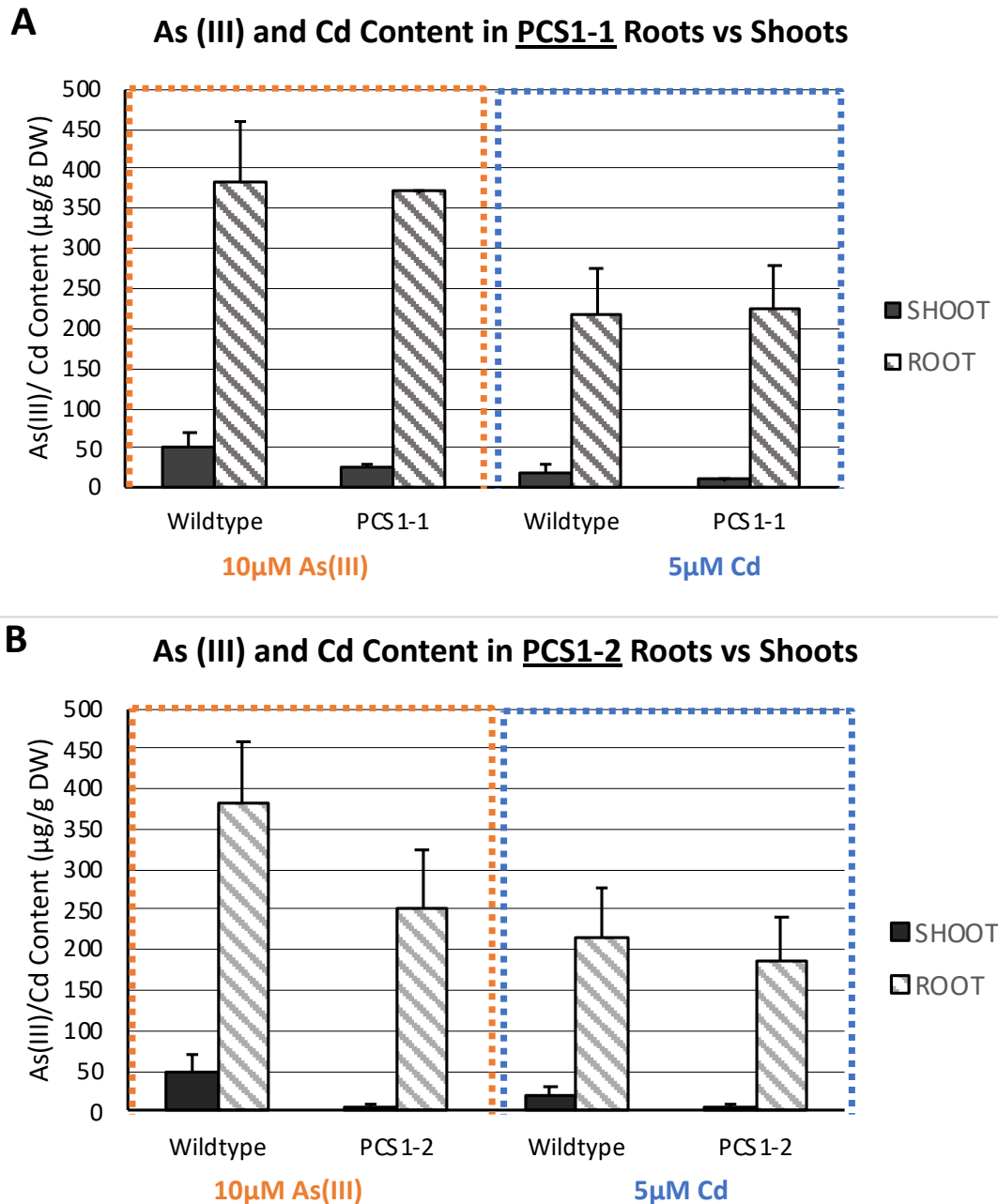**As (III) and Cd Content in <u>PCS1-2</u> Roots vs Shoots**

Figure 7: Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis measuring arsenite and cadmium content in shoot vs. root tissues of Nipponbare wildtype *and Triticum aestivum* phytochelatin synthase gene (TaPCS1) expressing rice lines. **A)** Shows results for the PCS1-1 transgenic lines. **B)** Shows results for the PCS1-2 transgenic lines. Rice seeds were germinated on water-soaked paper towels in Petri dishes for 7 days. Upon germination, seedlings were transferred to hydroponic chambers and treated with hydroponic nutrients (P_2O_5, K_2O, MgO, SO_3, N, K_2O, CaO, Cu, Fe, Mn, Mo, Zn) for 21 days. On day 22, healthy wildtype and transgenic TaPCS1 plants were treated with 10μM of As(III) and 50μM of $CdCl_2$ for 72-hours, where they were monitored for toxicity. Roots of treated plants were then washed with 1mM of K_2HPO_4 and 5mM of $CaCl_2$. Shoots and Roots were separated and dried at 37° C for 7 days. Tissue samples were digested in 70% Nitric Acid for ICP-MS measurements. Error bars represent SEM (n=6 for WT; n=3 for PCS1-1; n=2 for PCS1-2).

A   As (III) Content in Shoots of WT/PCS1-2

B   Cd Content in Shoots of WT/PCS1-2
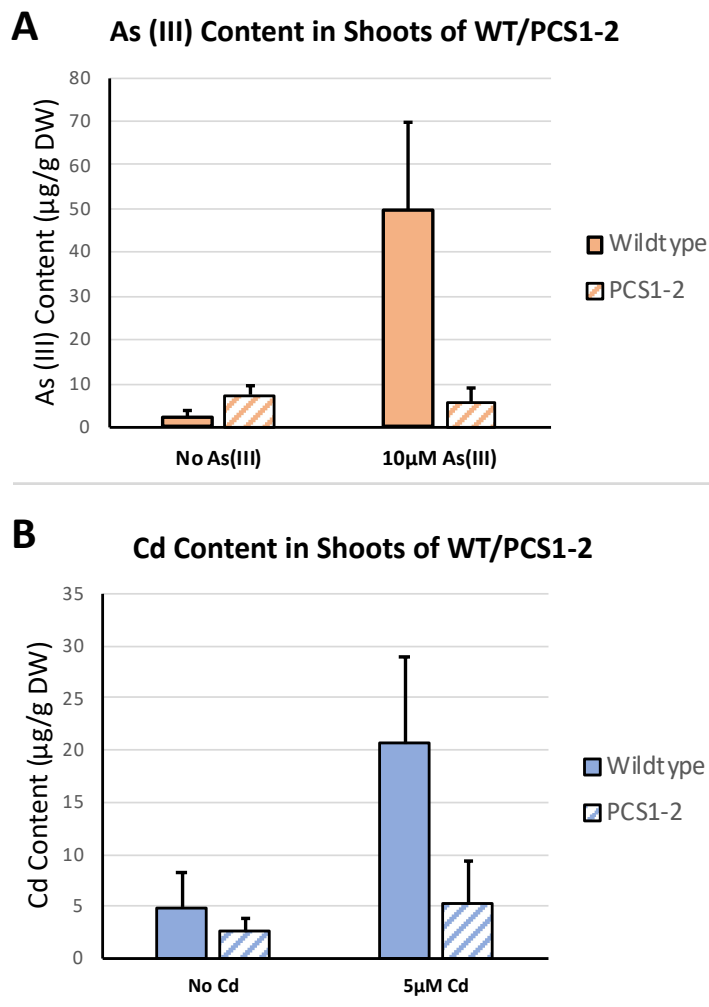
Figure 8: Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) analysis comparing cadmium content in shoot tissues of Nipponbare wildtype and transgenic TaPCS1 rice lines. Control conditions were exposed to hydroponic nutrients only. Experimental conditions were treated with 10μM of As(III) and 55μM of $CdCl_2$ for 72-hours. **8-A** shows the difference in As(III) accumulation between the shoot tissues of WT and PCS1-2. **8-B** shows the difference in Cd accumulation between the shoot tissues of WT and PCS1-2. Error bars represent SEM(n=6 for WT; n=2 for PCS1-2).

Discussion

1.1 AtHMA3

      Two rounds of heavy metal experiments were conducted with rice lines overexpressing AtHMA3 specifically in the roots. For the first round of experiments, rice plants were exposed to 55μM of cadmium for a total of 72 hours. Significant evidence of lower cadmium accumulation in the shoot tissues can be observed in the transgenic AtHMA3 rice lines when compared to the wildtype controls (Figure 4). However, there was no significant difference in root sequestration of cadmium between the transgenic lines and wildtype controls (Figure 2). Despite these findings, some limitations were identified in the first round of hydroponic experiments that were addressed for the second round of experiments. One such limitation was the concentration of cadmium at which rice plant lines were treated. At 55μM of Cd, a relatively high concentration can be causing unnecessary stress on select plants and leading to their toxicity. Plants experiencing such toxicity may contribute to noise in the data, as they can be outliers. A plausible explanation for the stress some plants are experiencing, despite overexpression of AtHMA3, could be that the HMA3 heavy metal pump is being oversaturated with Cd^{2+} ions due to the sudden exposure of high concentrations of cadmium. This can affect the functionality of the ATPase pump and hinder the efficiency at which cadmium ions are being sequestered into the vacuole. Thus, free-flowing cadmium ions are still able to translocate into other parts of the plant *via* the xylem where they can disrupt various biological processes.

      To take these limitations into consideration, the cadmium concentrations for the second round of hydroponic experiments were lowered from 55μM to 2μM. However, the duration of heavy metal exposure was kept consistent with the previous round at 72-hours. This reduction in cadmium concentration in the second round of experiments did not offer any significant results,

as the cadmium contents in the respective tissues of the wildtype and transgenic lines were similar (Figure 5). From this data, it can also be observed that the Cd contents in the shoot tissues of the wildtype lines are very low (~10μg/g). A plausible explanation for this could be that plants were not given enough time to translocate cadmium ions to their full potential due to the significant reduction in cadmium concentration and short duration of exposure (72-hours).

To address this limitation for the next round of experiments, future plans include increasing the duration of cadmium exposure from 72-hours to ~ 7-days. However, the plants will be treated with cadmium once again on the 4th day to keep the heavy metal exposure constant. Future plans also consist of repeating these experiments with recently obtained independent transgenic lines (28 lines) with root-specific promoters. Analyzing many independent lines will allow for the identification of lines with the strongest phenotypes. Rice lines will also be subject to various heavy metal exposure regimens consisting of altered Cd and As(III) concentrations and/or exposure durations based on what is observed with every step of experimentation. Furthermore, qRT-PCR will be utilized to measure expression of transgenes in independent rice lines to be compared to their observable phenotypes upon heavy metal exposure.

1.2 TaPCS1

Two rounds of hydroponics experiments were conducted with rice lines overexpressing TaPCS1 in root tissues with a root-specific promoter. The first round of hydroponic experiments was designed with heavy metal treatments consisting of 50μM of Cd and 10 μM of AS(III) for a total duration of 72-hours. The first round was able to provide some evidence of TaPCS1-2 overexpressing lines accumulating less cadmium in the shoot tissues when compared to wildtype

controls (Figure 6). However, As(III) uptake was relatively low when compared to the uptake of cadmium for both wildtype and PCS1-2 lines. This was possibly due to the relatively high concentration of Cd treatment (50μM). Since phytochelatins (PCs) are capable of forming complexes with both As(III) and Cd, exploring PCS1 in the context of As(III) phytostabilization also becomes difficult with a high concentration of Cd. An abundance of cadmium ions could outcompete As(III) in PC complex formation and possibly limit root sequestration of As(III).

To address this limitation in the second round of hydroponic experiments, the cadmium concentration was reduced to 5μM while keeping the concentration of As(III) at 10μM to promote As(III) uptake. Similar to the first round, the total duration of heavy metal exposure was 72-hours. The results of second round showed promising evidence of TaPCS1-2 lines accumulating less Cd and As(III) in shoot tissues when compared to WT controls (Figure 8). However, more experiments will be conducted with new treatment regimens to test this further. Moreover, more independent lines with root-targeted overexpression of TaPCS1 will need to be prepared for upcoming experiments. This will allow for identification of independent lines with strong phenotypes to be cross-checked with levels of transgene expression (qRT- PCR).

Previous literature has shown that phytochelatin (PC) bound heavy metal ions are further subject to vacuolar sequestration *via* a tonoplast-localized ATP-Binding Cassette transporter identified as Abc2 in *S. Pombe* and ABCC1/2 in *A. thaliana* (Mendoza-Cózatl et al., 2011). Research has also shown that these ABC transporters are capable of transporting both PC-Cd and PC-As(III) complexes into the vacuole (Mendoza-Cózatl et al., 2011). Since this ABC transporter plays a vital role in the phytoremediation approach this project aims to achieve, rice lines overexpressing *S. pombe* Abc2 transgene with a root-specific promoter have also been

prepared for future rounds of hydroponic experiments. Overexpressing SpAbc2 in the roots of rice will allow for further investigation of this transporter in the context of phytostabilization.

Understanding the behaviors of these individual transgenes (HMA3/PCS1/Abc2) when overexpressed in the roots of rice will contribute to the future of the project where combinations of these transgenes can be overexpressed in unison. Pairing these transgenes could potentially aid in the vacuolar sequestration of Cd and As(III); thus, further promoting root sequestration of these heavy metals. For example, overexpression of PCS1 promotes phytochelatin synthesis and the formation of PC-Cd and PC-As(III) complexes (Mendoza-Cózatl et al., 2011). However, it is possible that native expression of the tonoplast-localized ABC transporter does not allow it to keep up with the abundance of PC-Cd and PC-As(III) production with maximum efficiency. Therefore, overexpressing both PCS1 and Abc2 in the roots is one approach that can be implemented in the future of this project. Overexpressing both HMA3 and PCS1 is another approach that can be taken to allow for a collaborative effort in vacuolar sequestration and phytostabilization of cadmium. To properly plan for the most appropriate strategy, however, more experiments must be performed with several independent transgenic rice lines and exposure regimens in the immediate future of this project.

Materials and Methods

1.1 Lab Attire and Safety

All experimental procedures outlined below were performed in the appropriate lab attire while upholding laboratory safety protocols. Appropriate lab attire included but was not limited to a lab coat, nitrile safety gloves, and safety glasses. During the COVID19 pandemic face masks and social distancing were implemented together with regular UCSD PCR testing. Where appropriate, procedures were performed under a fume/sterile hood to ensure safety and accuracy.

1.2 Obtaining Transgenic Rice Lines

Transgenic rice lines overexpressing wheat phytochelatin synthase gene (TaPCS1) *Arabidopsis thaliana* Heavy Metal Associated3 gene (AtHMA3) were prepared by Dr. Gvheung An at Kyung Hee University, Korea. Plasmid vectors (pGA4082) used for the transformation rice lines were designed with a root-specific promoter (*peroxidase40*). Rice lines were genotypes prior to further experimentation by extracting DNA from leaves and performing Polymerase Chain Reaction (PCR) with primers appropriately designed for gene of interest (Table 1).

1.3 Seed Sterilization

In order to prepare rice seeds for germination, they were first sterilized to avoid any further contamination. Seed sterilization was performed under a sterile fume hood. Around 15-20 seeds of the same genotype were placed in sterile 50mL Falcon conical tubes. For the first wash of the seeds, 10mL of 70% percent ethanol solution was added to each tube. Tubes were vigorously shook for approximately 60 seconds and the ethanol solution was carefully discarded from the

tubes. For the second wash of the seeds, 10mL of 50% commercial bleach solution was added to each tube. The tubes were then placed on an incubation shaker for 30 minutes on the lowest setting. After the 30-minute period was complete, the bleach solution was discarded from the tubes. The seeds were rinsed with 10mL of deionized water a total of 5 times. The seeds were then poured out of the conical tube and onto sterile filter paper where they were allowed to dry.

1.4 Rice Germination Conditions

Rice seeds were germinated on water-soaked paper towels in sterile Petri dishes. A total of 10-12 seeds were allocated to each Petri dish. Seeds were scattered across the paper towel using sterile forceps to ensure that each seed had enough room to germinate and grow without interference from neighboring seeds. Each Petri dish was then sealed using rubberized waterproof tape and stored at room temperature for a total of 7 days, or longer depending on the state of the root growth. Once there was sufficient root growth, seedlings were scanned for contamination and health. Competent seedlings were transferred to hydroponic chamber systems using sterile forceps.

1.5 Hydroponic Growth Conditions

Rice plants were grown hydroponically for a total of three weeks in a growth room under a 16-h/8-h light/dark cycle at ~ 21°C. During the three-week growth period, plants were exposed to a 3-part hydronic nutrient system called the "Flora Series" from General Hydroponics consisting of the following nutrients: P_2O_5, K_2O, MgO, SO_3, N, K_2O, CaO, Cu, Fe, Mn, Mo, Zn. The "Flora Series" includes three different bottles of nutrients: FloraBloom, FloraMicro, and FloraGro. Hydroponic media was prepared by adding 1mL of each of the aforementioned bottles of

nutrients to 1.8L of water. This media was completely replaced every 4 days. In order to create a humid environment for the plants, hydroponic systems were placed in deep autoclave trays and completely sealed with saran wrap for the interim of their growth period. After the three-week hydroponic growth period, plants were either transferred to soil for further propagation in a greenhouse or kept in the hydroponic systems for heavy metal and metalloid treatments .

1.6 Greenhouse Growth Conditions

Rice plants that needed to be further propagated were transferred from hydroponic systems and potted into 5-gallon buckets consisting of rice potting soil. These plants were grown in greenhouse conditions following a natural light/dark cycle at room temperature (~27°). Rice plants were watered every 4-days to ensure that the buckets containing the rice plants were completely flooded at all times.

1.7 Heavy Metal Treatment Conditions

Upon three weeks of hydroponic growth conditions, select rice plants were hydroponically exposed to heavy metal treatment under the same growth room conditions. Prior to heavy metal treatment, unhealthy plants were removed from the hydroponic system and properly discarded. Transgenic rice plants and wildtype control were then exposed to either cadmium ($CdCl_2$) or both cadmium and pure arsenite (As(III)), depending on the gene being overexpressed by the respective plant line. Plant lines expressing *Arabidopsis thaliana* Heavy Metal ATPase (AtHMA3) were exposed to different concentrations of cadmium. Plant lines expressing wheat phytochelatin synthase gene (TaPCS1) were exposed to different concentrations of both cadmium and arsenite. Media for heavy metal treatment conditions consisted of the heavy

metal(s) of interest (Cd/As(III)) and the previously mentioned hydroponic nutrient solution

(P_2O_5, K_2O, MgO, SO_3, N, K_2O, CaO, Cu, Fe, Mn, Mo, Zn), whereas the media for control

conditions consisted solely of the hydroponic nutrient solution. For the first round of treatment,

AtHMA3 rice lines and wildtype controls were exposed to 55µM of cadmium for a total of 72

hours, and TaPCS1 rice lines and wildtype controls were exposed to a mixture of both 50µM of

cadmium and 10µM of arsenite for a total of 72 hours. For the second round of treatment,

AtHMA3 lines were exposed to 2µM of cadmium for a total of 72 hours, and TaPCS1 lines were

exposed to both of both 5µM of cadmium and 10µM of arsenite for a total of 72 hours. Plants

were closely monitored to ensure that they were harvested prior to experiencing toxicity.

1.8 Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Sample Preparation

Upon exposure to the appropriate treatment for a total of 72-hours, plants were individually

extracted from the hydroponic system using sterile forceps. Immediately upon extraction, a wash

was performed on the roots of each rice plant. The roots of plants that were exposed to cadmium

were washed with 5mM $CaCl_2$ solution. The roots of plants that were exposed to both cadmium

and arsenite were washed with 1mM of K_2HPO_4 and 5mM of $CaCl_2$. Each root was taken

through a series of three separate washes, with each wash being approximately 60 seconds. The

wash was performed by carefully handling the plant using sterile forceps, dipping the root in the

beaker containing the wash solution, and continuously rinsing the root in the solution. After the

three washes were complete, the roots were rinsed with sterile deionized water. Following the

wash, the root and shoot tissues of each plant were separated using sterile medical grade scissors

and placed in separate Falcon conical tubes. Plant tissues were then dried in an incubation room

at 37°C for a total of 7-days. The dry weights of each root/shoot tissue sample were measured

and recorded for data analysis. Tissue samples were digested in 3mL of 70% Nitric Acid under the fume hood for 24 hours. Sample containing tubes were then placed in boiling water for 30 minutes to completely boil the samples. The samples were centrifuged at 3,000 rpm for 25 minutes. After the centrifugation was complete, a pipette was used to transfer 1mL of the supernatant of each sample to sterile 15mL Falcon conical tubes. Each sample was diluted with 2mL of sterile deionized water. The samples were shipped to the Donald Danforth Plant Science Center where Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was used to measure arsenite and cadmium contents in each tissue sample.

1.9 ICP-MS Data Analysis

ICP-MS data provided by the Donald Danforth Plant Science Center was presented in an excel sheet with numerical values for each element found in each tissue sample (root/shoot). The units of these values were Part-Per-Billion (PPB). Each value was divided by the dry weight (in grams) measured previously and multiplied by 0.003 due to 3X dilution to achieve units in the form of **ug/g**. Averages for each set of replicates were obtained to create bar graphs comparing cadmium and arsenite contents in root/shoot tissues between transgenic and wildtype lines. Error bars were obtained by calculating the Standard Error of the Mean for each set of replicates. All data analyses were performed using Microsoft Excel for Mac (2021). Statistical analyses were performed in the form of T-test and One-way ANOVA/Tukey's test to check for statistical significance.

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