Root to Shoot: Zinc Translocation Increases with Mutation in HMA3 in *Setaria viridis*

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ABSTRACT

Heavy Metal ATPase 3 (HMA3) is an enzyme that mediates zinc and cadmium influx to root cell vacuoles. Research in phytoremediation suggests that mutations in this enzyme can increase above ground localization of these ions, thus optimizing plants for phytoextraction of zinc and cadmium from soils where they exist in excess. *Setaria viridis* is grass that can be used in phytoremediation as a result of its ability to tolerate growth in high levels of zinc. This paper investigates the impact on plant morphology and physiology caused by a knock-out mutation in HMA3 in *S. viridis.* Exploring the impact of this mutation was first attempted through the CRISPR-Cpf1 system but failed at the regeneration phase of the study. The study is currently being attempted again with CRISPR-Cas9 through this ongoing experiment. After transformation and regeneration, 40 plants will be harvested to assay size, dry-weight, and zinc content; 10 mutants will be grown in elevated zinc concentrations and 10 will be grown in normal ion concentrations. An equal number of wild-type plants will be grown in the same treatments as controls. I hypothesize that a mutation in HMA3 will create a statistically significant difference in zinc translocation from the roots to shoots in mutants compared to wild-type plants. I also anticipate HMA3 mutants in high zinc concentrations showing reduced biomass accumulation and mild chlorosis. Overall, this paper will provide novel insights into a little-studied, emerging model system for research in monocot plants with the goal of engineering *S. viridis* for use in phytoremediation.

KEYWORDS

Phytoremediation, *Setaria viridis*, zinc, Heavy Metal ATPase 3, CRISPR

INTRODUCTION

Over the last two hundred years, human activity has contaminated soils throughout urban and rural areas, necessitating a practical solution for heavy metal remediation. This widespread soil pollution is conducive to numerous, chronic health issues even at very low (e.g. μg/L) concentrations (UWE Bristol, 2013). Lead, a notoriously toxic heavy metal used widely in the 20th century, is responsible for high lead soil concentrations in urban areas, sourced from leaded gasoline burning cars and from houses built with lead paint (Mielke et al. 1997). Lead toxicity at 10 μg/dL concentrations in blood irreversibly decreases childhood IQ by 8 points (Lanphear et al. 2005). Similarly, cadmium also has devastating health affects in afflicted individuals. In the well-studied emergence of "itai-itai" disease in 1912 Japan, people suffered from cadmium poisoning by consuming rice watered with contaminated mine drainage. Victims suffered from brittle bones, anemia, multiple organ failure, and eventually death (Chaney et al. 1999) Although cadmium poisoning on the scale of itai-itai disease has not recurred, cadmium soil contamination persists in the United States due to industrial practices which also lead to high rates of zinc deposition (Holmgren et al. 1993). Zinc and cadmium are also absorbed into plant cells through the same pathways (Lin and Aarts, 2012). In cases where zinc exists in excess, plants, soil microbes, and arbuscular mycorrhizal fungi all experience decreased growth, reflecting that heavy metals and their widespread contamination make the development of a cost-effective remediation solution imperative.

Although there are numerous approaches to address soil contamination, an increasingly viable and cost-effective technique, phytoremediation, has gained momentum as a panacea for low to moderate levels of soil contamination (Sarma 2010). Phytoremediation is the cultivation of heavy metal hyper-accumulating plants on heavy metal contaminated soils with the intent of harvesting the plants to remove heavy metals from the soils. Traditional soil remediation methods can cost up to \$300/m^3, yet phytoremediation can drive remediation costs down to \$0.05/m^3 (Cunningham et al. 1997). In a study on chromium removal from wastewater with water hyacinth, 99.5% of chromium was removed from contaminated drainage in 15 days (Saha et al. 2017). With promising results in non-transgenic plants, implementing CRISPR may unveil a key to swiftly addressing environmental heavy metal contamination.

In high concentrations, zinc limits the growth of numerous plant species, but a select few are able to tolerate very high heavy metal concentrations (Sarma 2010). Through root system secretion of phytochelatins—small tri-peptide molecules—by phytochelatin synthase, plants can fix and absorb heavy metals from soils for sequestration (Vatamanuik et al. 1999). Once in the roots, if in excess, heavy metals such as zinc are transported to the root vacuoles for plant cell detoxification (Mendoza-Cózatl et al. 2011). Subterranean heavy metal localization presents a limiting factor to phytoremediation efforts, because it is easier in practice to remove heavy metals from a contaminated site, if the heavy metals are localized in the shoots and leaves.

HMA3 is a gene involved in zinc and cadmium translocation (Mendoza-Cozatl et al. 2011). HMA3 belongs to a diverse family of P_{1B}-type ATPases, which include HMA enzymes. HMA enzymes are involved in plant responses to heavy metal stresses and are localized in diverse locations throughout plants (Takashi et al. 2012). This enzyme has the highest expression in the tonoplast of root cells and has two primary domains responsible for generating an electromagnetic gradient through phosphorylation by ATP, which transports zinc and cadmium ions from the cytoplasm to the vacuole (Wang et al. 2014). Further understanding this gene's role by growing transgenic *hma3* mutants (plants that lack a functional HMA3 gene) in soils with elevated zinc concentrations will provide valuable information on plant responses to elevated ion concentrations above the roots, thus addressing gaps in research, where there is a lack of information on the impact of mutations in HMA genes on ion translocation and plant development.

HMA3 can be interrogated using genome engineering, specifically, CRISPR. With CRISPR, researchers can now investigate—and even augment—the phytoremediation potential of virtually any plant species. Prior to the development of the CRISPR-Cas system, highly specific genetic research exploring the roles of specific genes was cumbersome and not easily feasible (Ding et al. 2016; Porteus and Carroll 2005; Sanjana et al. 2012). With CRISPR-Cas, relatively few inputs are needed when compared to older systems such as Zinc Fingers and TALENs (Porteus and Carroll 2005; Sanjana et al. 2012). Zinc Fingers rely on a synthetically designed enzyme and a naturally occurring restriction enzyme that can cut DNA at only one type of recognition site; this combination can induce mutagenesis at a specific location, but it is more costly than simply inserting a guide RNA of any design that directs a more robust enzyme to any site in the genome (Porteus and Carroll 2005, Ding et al. 2016). TALENs work in a similar way as Zinc Fingers, but they rely on long chains of proteins that bind to a single nucleotide at a time, which becomes more

expensive as a target site becomes longer and more TALENs are linked together (Sanjana et al. 2012).

Using the CRISPR-Cas genome engineering system, it is nowpossible to increase root to shoot heavy metal translocation by knocking out a gene involved in root vacuolar heavy metal sequestration. CRISPR-Cpf1 is a newer CRISPR system that demonstrates greater efficiency at generating biallelic mutations in T_0 mutant lines than its earlier Cas9 variant (Tang et al. 2017). With this system, specific mutations in *Setaria viridis* can be used to effectively expedite the time it takes to perform genetic research in plant biology. Unfortunately, attempts at mutagenesis with Cpf1 proved unsuccessful and I am now conducting my research using CRISPR-Cas9.

My thesis aims to examine the impact of a mutation in HMA3 in *S. viridis* with respect to growth in different concentrations of zinc. Specifically, I seek to determine the differences in morphology, biomass accumulation, and zinc concentrations in the roots and leaves between wild type and *hma3* mutants, both grown in soils with elevated zinc concentrations. Once plants are ready to be harvested, they will be partitioned into roots and leaves, and measured for size and dry-weight. Each partitioned section will then be incinerated through a method known as dry-ashing, which will allow me to quantify their zinc concentrations. Concentrations will be assessed through mass-spectrometry. The differences in zinc concentrations, plant morphology, and biomass accumulation between wild-types and *hma3* mutants will then be statistically analyzed. Assessing multiple variables in tandem will provide valuable insights on the impact of knocking-out the HMA3 enzyme in *S. viridis*. I hypothesize that *S. viridis* will be a successful transgenic phytoremediation candidate.

METHODS

Setaria viridis

To select my plant species, I focused on determining which plant species are not recurrent in phytoremediation research. Literature overwhelmingly favor research in dicot species over monocot species (Sarma 2010). This is interesting, as monocot species represent the majority of agricultural productivity through rice, wheat, and maize. *Setaria viridis,* more commonly known as wild foxtail millet, is grass native to Eurasia but can be found on every continent except for

Antarctica. It is the wild ancestor of *Setaria italica* and is therefore adapted to growth in a greater range of environments (Muthamilarasan and Prasad 2017). Research on the heavy metal uptake properties of *S. viridis* is limited; however, the genome of *S. viridis* has been fully sequenced, making it a good model organism for phytoremediation research in monocots. To conduct my experiment, I will use the *S. viridis* accession Me034.

Genome Engineering with CRISPR-Cas9 and CRISPR-Cpf1

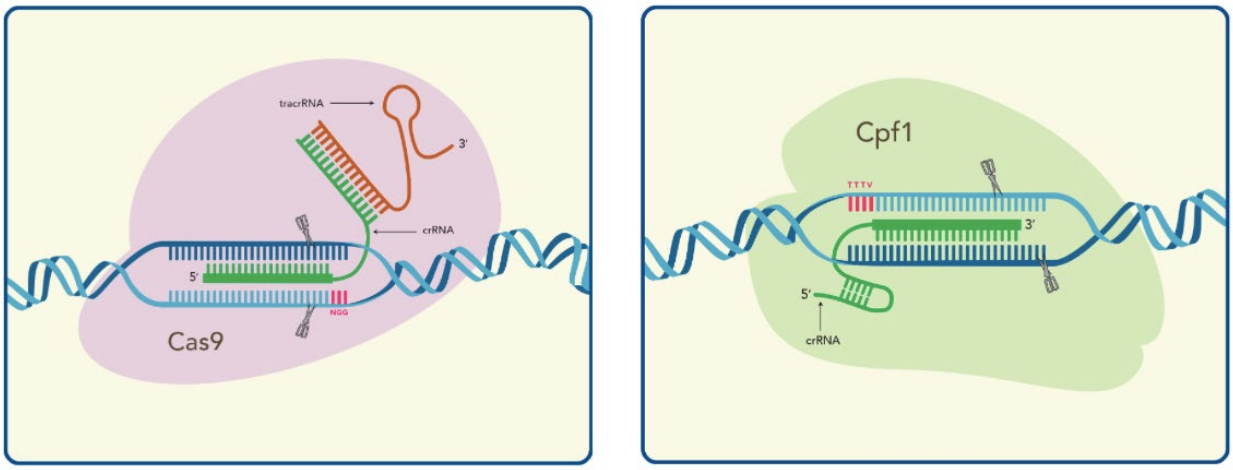

Figure 1: A diagram illustrating how the Cas9 and Cpf1 enzymes identify and mutagenize a location in the genome. Provided by Integrated DNA Technologies Inc.

To create a Heavy Metal ATPase 3 (HMA3) *S. viridis* mutant, I first employed the CRISPR-Cpf1 genome engineering system and then the CRISPR-Cas9 system (Tang et al. 2017; Ding et al. 2016). CRISPR-Cpf1 and –Cas9 refers to three separate terms. CRISPR is an acronym for "Clustered Regularly Interspaced Short Palindromic Repeats," which refers to short, recurring sequences in a target genome that allow the Cpf1 and Cas9 enzymes to attach themselves to a target sequence and cleave DNA. CRISPR-Cpf1works as follows: first, Cpf1 must recognize a 5'-TTTV-3' (V = C, G, A) protospacer-adjacent motif (PAM) within a target sequence. Second, with a pre-designed 20 to 23-nucleotide guide RNA (crRNA), Cpf1 is guided to the site for mutagenesis by Watson-Crick base pairing with its complement sequence in the target genome. This mutagenesis only works if the guide RNA is designed such that its complement is immediately downstream of a PAM in the genome sequence. Once at its target site, Cpf1 is able to create a double-stranded DNA break and induce a knock-out mutation (Tang et al. 2017). CRISPR-Cas9

works similarly except, the Cas9 PAM is 5'-NGG-3' (N = C, G, A, T), uses a 20-nucleotide guide RNA whose complement is immediately upstream from the PAM, and uses a tracrRNA in addition to the crRNA (Ding et al. 2016).

A notable advantage that Cpf1 has over Cas9 is that when it cleaves DNA, it cuts DNA such that it creates "sticky-ends" which leaves one strand of DNA longer than the other on both ends. This enhances the efficiency of genetic insertions and homology directed repair (Tang et al. 2017). Cpf1 also preserves the PAM because the enzyme cuts further away from the motif than Cas9 (Figure 1). Cas9 creates "blunt end" cuts, where both ends of the cuts have DNA strands of equal length, this is a less efficient method for gene insertion, because inserted DNA cannot recombine as efficiently (Ding et al. 2016). Cas9 also destroys the PAM during cleavage, which makes repeated mutagenesis at the same site impossible (Integrated DNA Technologies Inc).

Site-Directed Mutagenesis

To mutagenize *S. viridis* with Cpf1, I had to assemble the Cpf1 plasmid from its smaller component plasmids. I first created a Cpf1 plasmid vector with my guide RNA, inserted it into *Agrobacterium tumefaciens*, and applied the *A. tumefaciens* to *S. viridis* calli. For Cas9 plasmid construction, I ordered a complete Cas9 plasmid with my guide RNA from Integrated DNA Technologies Inc. I did this because Cpf1 did not work and time is a constraint. Ordering a completely premade construct is more expensive than assembling a construct, which is the reason why I did not order the premade construct for Cpf1 when I initially believed it would work.

I first designed three guide RNAs directly downstream from three different 5'-TTTV-3' PAMs in my target sequence. I then performed a ligation with my guide RNA inserts to pYPQ141C vector plasmids provided by Addgene NPO, following New England Biolab's T4 DNA Ligase protocol (neb.com). To calculate the appropriate molar concentrations between my vector and inserts, I used the NEBioCalculator provided by NEB online (nebiocalculator.neb.com). After ligating my inserts and vector into their respective ligation reactions in PCR tubes, I performed three bacterial transformations. Transformations were conducted following an online protocol provided by Addgene. Bacterial transformations, also known as cloning, insert ligated plasmids into *Escherichia coli* cells, which are then used to replicate more plasmids after they are transformed.

To ensure that cultured *E. coli* cells had my plasmid construct, they were cultured on six liquid broth (LB) agar petri dishes with a 1% concentration of spectinomycin. Each transformation was plated on two petri dishes, one dish had 166 µl of *E. coli* and growth media plated and the other had 83 µl. The plasmid contains a gene encoding for spectinomycin resistance, meaning that whatever grows on the LB agar plate has the plasmid encoding for spectinomycin resistance and the Cpf1 guide RNA. After cloning, I selected colonies to grow for plasmid extraction.

I selected three colonies from each petri dish, except for one that was unusable, to grow in 3 mL of 1% spectinomycin aqueous LB. I then incubated the liquid culture at 37ºC for 16 hours. I then prepared the 15 colonies for plasmid extraction by harvesting 1 mL of liquid culture. I performed the plasmid extractions using an AccuPrep® Plasmid Mini Extraction Kit from Bioneer. I performed fifteen total plasmid extractions. Extracted plasmids were then sent to Quintara Biosciences for sequencing. Fourteen of the fifteen sequenced plasmids had a guide RNA in the appropriate location, indicating 14 successful bacterial transformations.

I then performed a Golden Gate assembly using six successful plasmid extracts. For this transformation, each gRNA plasmid was ligated to a plasmid containing the Cpf1 enzyme and a binary plasmid. The reactions were performed in 6 PCR tubes with 5µl of reagents per tube. Each tube had 100ng of Cpf1 plasmid, 100ng of gRNA plasmid, 200ng of binary plasmid, 1µl of LR Clonase, 0.1µl of Proteinase K, and the remainder of the 5µl was supplemented with autoclaved deionized water. Tubes were then incubated overnight at 25°C. I then performed a bacterial transformation with the Golden Gate assembly plasmids using the same transformation protocol mentioned above.

To verify successful Golden Gate cloning, I ran a restriction digest with two restriction enzymes that cut the golden gate plasmid into its three component plasmids on a gel using the restriction enzymes, EcoRI and PTS1. I saw three separate bands on my gel, indicating that cloning was successful. Finally, I performed another bacterial transformation with my golden gate plasmid and verified successful *E. coli* transformation with PCR and a restriction digest. To ensure that I had more *E. coli* cells that were successfully transformed from the same colonies, I created a glycerol stock of *E. coli* that was 50% glycerol and 50% LB that had a 1% concentration kanamycin with *E. coli* from growing in it. The golden gate plasmid encoded kanamycin resistance, instead of spectinomycin resistance.

Setaria viridis **transformation**

To prepare for *A. tumefaciens* transformation, I grew out the glycerol stock *E. coli* at 37ºC for 16 hours in LB with a 1% concentration of kanamycin. I then performed a plasmid extraction following the AccuPrep® Plasmid Mini Extraction Kit (Bioneer). I then performed an *Agrobacterium* transformation (G. Chuck, *unpublished lab protocol*). *Agrobacterium tumefaciens* has a unique operon that enables the bacterium to bypass plant cell walls and insert foreign DNA (i.e. the Cpf1 or Cas9/gRNA plasmid) into plant nuclei (Stachel and Zambryski 1986). I plated the transformed *A. tumefaciens* cells on LB-agar petri dishes with 1% carbenicillin and 1% kanamycin concentrations. I incubated them for 3 days at 28ºC. After 3 days, I selected colonies to grow in 3 mL aqueous LB for another three days. I then created a glycerol stock of *A. tumefaciens* cells with 500 µL of the liquid *A. tumefaciens* culture and 500 µL of 50% glycerol. I then performed a plasmid extraction using 1 mL of the liquid *A. tumefaciens* culture with an AccuPrep® Plasmid Mini Extraction Kit from Bioneer. I verified successful transformation with PCR and restriction digest.

To transform *S. viridis*, I applied the *A. tumefaciens* cells to *S. viridis* calli. Calli (singular *callus*) are small, undifferentiated globs of plant cells (Ikeuchi et al. 2013). After *A. tumefaciens* application, calli were incubated at 4°C for four days. I washed the calli with deionized water on the fourth day and placed them on new petri dishes with phytagel, auxin, and cytokinin. I returned the calli to a 4°C refrigerator for one week. At the end of that week, I selected 50 calli to grow into fully differentiated *S. viridis* plants. Regeneration was achieved through plant growth hormone application of auxin, cytokinin, and gibberellic acid. After three weeks of regeneration, I attempted to verify successful *S. viridis* transformation, but my results came back negative for transformation after using PCR to look for an HMA3 knock-out. After consulting with other members of the Hake Lab, I decided it would be best to reattempt mutagenesis with Cas9.

To save the time it took to put together the final Cpf1 construct, I ordered the complete Cas9 construct from Integrated DNA Technologies Inc. I will clone this Cas9 construct into *E. coli* and into *A. tumefaciens* through the same methods mentioned above. This transformation will take 2 weeks. After retransforming *Agrobacterium* with the new Cas9 construct, I will prepare calli for transformation through the same methods as mentioned above.

Experiment Site and Execution

To complete my experiment, I will use the growth chambers at the USDA Western Regional Research Center in Albany, CA. The growth chambers operate on a 12-hour light-cycle at 25ºC. The plants will be grown on a powder clay aggregate, provided by collaborators of the Hake Lab. The clay aggregate is easier to remove from roots than regular soil. Zinc will be mixed in with the growth medium as $ZnSO_4$ powder and shaken in a sealed container.

All calli from the Me034 accession will be regenerated. The presence of a mutation will be genotyped through PCR. 40 of the successful regenerates will be used for the experiment.

40 plants total will be grown in the control and zinc treatment. All plants will be grown in their own pots. 10 homozygous HMA3 mutants and 10 wild-type *S. viridis* siblings from the same accession, Me034, will be grown in 100 mg kg^{-1} $ZnSO_4$ mixed with clay aggregate provided by the Harmon Lab (Kaznina et al. 2009). A homozygous mutation will be assayed with PCR. As a control, I will grow 10 HMA3 mutants and 10 wild type plants in growth medium with no zinc.

To harvest plants, I will wait until they reach the 5-leaf stage. At this stage in the *S. viridis* life cycle (2 weeks), all plants will likely still be growing regardless of toxicity. This is because maternal nutrients from the seed are getting depleted at this point (B. L. Haining, *personal communication*). Therefore, it will be a good time to harvest the plants for analysis, because the zinc toxicity will not yet be affecting vigor to a significant extent and plants will still be transporting it zinc (B. L. Haining, *personal communication*).

Harvest and Analysis

I will harvest plants by gently removing them from the medium and washing off the clay aggregate with deionized water. I will then partition plants into roots and leaves. I will first measure the lengths of each partition, then measure plants for their dry-weight. To determine dry-weight biomass accumulation, I will place the plants in a seed-dryer for two days at 60°C. I will then weigh each partition. After drying, all samples will be sent to collaborators of the Hake Lab for zinc assays in the roots and leaves.

Data Analysis

To interpret the data, I will implement t-tests and two-way ANOVA. I will first measure the parameters in wild-types and mutants: root weight, leaf weight, root size, leaf sizes, and zinc concentrations in the roots and leaves. I will then perform T-tests on all recorded parameters to assay if a homozygous HMA3 mutation had a statistically significant impact on my variables of interest compared to the wild-type plants. To discern relationships between treatment and genotype, I will perform multiple two-way analyses of variance. I will perform two two-way ANOVA on root weight and leaf weight as my dependent variables, and use wild-types and mutants and high zinc and normal zinc treatments as my independent variables. Similarly, I will also look at root and leaf zinc concentrations with respect to treatment and genotype. Last, the fifth and sixth ANOVA will be computed using leaf size and root size as the respective dependent variables.

DISCUSSION & PROGRESS TO DATE

Although CRISPR-Cpf1 appeared to be a very promising tool for genetic research, it currently does not work in *Setaria viridis.* This finding has forced me to continue my research using the longer-established CRISPR-Cas9 system.

CRISPR-Cas9 & CRISPR-Cpf1

CRISPR-Cas9 and CRISPR-Cpf1 have both demonstrated success at mutagenesis in a variety of plant species. From the literature, CRISPR-Cpf1 appeared to hold higher potential in the *S. viridis* system, but both through my results and in consultation with others, I learned that it does not yet work in *Setaria*. Researchers in the Hake Lab and outside labs all have difficulties mutagenizing *S. viridis*, so I have reverted to Cas9 to test the effects of HMA3 mutagenesis on Zinc translocation following the same transformation protocols.

CRISPR-Cpf1 may have failed because the incorrect U6 promoter was used. After starting over and redesigning a construct to fit Cas9's parameters, one thing that stood out to me was the promoter used to activate CRISPR. Promoters are short sequences that trigger gene transcription. To activate CRISPR, the RNA Polymerase III U6 promoter is needed, and each plant species has

a unique U6 promoter (Huang et al. 2016). The United States patent on Cpf1 does not include the *S. viridis* U6 promoter; the Cpf1 construct to mutagenize *S. viridis* relies on the U6 promoter from *Triticum aestivum*, or, wheat (Begemann and Gray 2016). Past experiments with Cas9 and *S. viridis* have used the same *T. aestivum* promoter with success (Huang et al. 2016). If the Cpf1 plasmid had the *S. viridis* U6 promoter, mutagenesis could have been successful. In the future, I would like to design a Cpf1 construct with the appropriate U6 promoter to test this hypothesis in *S. viridis.* Until then, I will use the Cas9 construct.

Zinc Translocation and *Setaria viridis*

HMA enzymes share very high homology in their structure and function. In a study on the roles of HMA2, HMA3, and HMA4 in *Arabidopsis thaliana*, researchers only speculated that HMA3 played a role in zinc localization (Hussain et al 2004). They were limited in their ability to assay HMA3 because of its close proximity to HMA2 on the same chromosome, and using recombinant DNA instead of CRISPR, they could not target both genes. HMA2 and HMA4 are localized in the plasma membrane of plant cells with higher expression levels in the roots; they mediate ion fluxes out of cells for numerous heavy metals (Satoh-Nagasawa et al. 2012; Hussain et al. 2004). For the role of HMA3 in *Oryza sativa* a mutation causes increased cadmium translocation from the roots to shoots (Miyadate et al. 2011). HMA3 transports zinc and cadmium, so it is plausible to infer zinc would also be transported above ground, especially given that HMA2 and HMA4 also transport zinc (Park et al. 2012; Ueno et al. 2011, Zhang et al. 2018). HMA3 is heavily expressed in the tonoplast of root cells and mediates zinc and cadmium influx to the vacuole. HMA3 and the other HMA enzymes are closely related to each other and largely homologous, suggesting that they all resulted from retroactive gene duplication events in the past (Nosil and Schluter 2011).

There is only one study on *Setaria viridis* responses to increased zinc. Kaznina et al. (2009) found that zinc concentrations of 80 mg kg^{-1} had no significant effect on *S. viridis* growth. At concentrations above 160 mg kg^{-1}, *S. viridis* begins to show reduced biomass accumulation and chlorosis, implying adverse effects from toxicity indicating promise for using CRISPR to increase above ground zinc concentrations for phytoremediation. Based on literature on HMA3, I can infer that a mutation is characterized by increased root to shoot zinc translocation in *S. viridis.* Studies

in rice, *Oryza sativa,* and *Arabidopsis thaliana* and *A. halleri* support HMA3 playing a role in mediating zinc influx to the vacuole (Miyadate et al. 2011; Park et al. 2012). With respect to phytoremediation, where information on *S. viridis'* potential is sparse (Krause and Kaiser 1970; Sarma 2010; Kaznina et al. 2009). Furthermore, I can also infer, because *S. viridis* and *S. italica* are so closely related, that the information I generate from studying zinc uptake in *S. viridis* will be highly transposable to *S. italica*. This may be important for studies on zinc and *S. italica*, where enhancing *S. italica's* nutritional value would be of interest.

Limitations

My study on HMA3 mutagenesis for zinc translocation in S. viridis has been limited by successful mutagenesis, time, and zinc sampling. Failure to implement Cpf1 in the study presented the greatest setback. To counter this, plasmid constructs were redesigned to fit Cas9's parameters. I initially wanted to grow my plants until they reached the F2 generation to obtain higher rates of homozygosity for a mutation in HMA3, but a short timeframe prevented this. I also wanted to measure the amounts of zinc in the seeds of *Setaria* as well. This could have been achieved if time allowed for the parental generation to flower and produce seed. Millet seeds are harvested for consumption in *S. italica*, so being able to quantify how much zinc accumulation increases in *S. viridis*, an extremely closely related ancestor, seeds would provide useful information on *S. italica*'s potential for transgenic biofortification.

Future Research

HMA3 plays a role in regulating zinc translocation from the roots to shoots of *S. viridis* by mediating zinc efflux from the cytoplasm to vacuoles of root cells (Park et al. 2012). These findings are supported by other research on HMA enzymes (Ueno et al. 2011, Zhang et al. 2018). To further explore optimizing plants for phytoremediation, I would look at the interactions of HMA enzyme mutations with gene expression through transcriptomics. Differential gene expression in wild-type plants and mutants, RNA-seq (RNA sequencing) on the roots and leaves of mutants and wild-types would determine which genes are being differentially expressed in mutant and wild-type plants (Wang et al. 2009). This information would allow me to choose which genes to further study in

tandem with a mutation in HMA3. Protein families such as Zinc Induced Proteins (ZIP) and Cation Diffusion Facilitators (CDF) play important roles in zinc tolerance and uptake (Grotz and Guerinot 2006; Ramesh et al. 2003; Burleigh et al. 2003). With data from RNA-seq and literature, I could effectively pick another gene to improve zinc uptake and tolerance by knock-out or knock-in using CRISPR. With research in plant biology practically exploding as technology rapidly improves, the potential for transgenic improvement in *Setaria* is more possible than ever.

Implications

Understanding the impact of a mutation in HMA3 in *S. viridis* will provide previously unknown insights into how the plant and its domesticated descendant, *Setaria italica* may fair as a phytoremediators and nutritionally fortified subsistence cultivars, respectively. Genome engineering with CRISPR provides an invaluable tool to begin exploring this possibility. With increasing research on other plant species and protein families that have closely linked roles to micronutrient acquisition, the stage is set for more research in *S. viridis* on this gene coupled with mutations in families such as CDF and ZIP. The quick, perennial growth, of *S. viridis* should be able to shed light on questions in phytoremediation, specifically, if zinc removal can be accelerated with a mutation in HMA3.

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