Generation and Characterization of Transgenic Chlamydomonas ReinhardtII Overexpressing the Novel Gene CIA7

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GENERATION AND CHARACTERIZATION OF TRANSGENIC CHLAMYDOMONAS
REINHARDTII OVEREXPRESSING THE NOVEL GENE CIA7

A Thesis

by

JUAN CARLOS ONOFRE RENTERIA

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

May 2020

Major Subject: Biology
GENERATION AND CHARACTERIZATION OF TRANSGENIC CHLAMYDOMONAS REINHARDTI I OVEREXPRESSING THE NOVEL GENE CIA7
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ABSTRACT

Generation and Characterization of Transgenic *Chlamydomonas reinhardtii* overexpressing the novel gene CIA7 (May 2020)

Juan Carlos Onofre Renteria, B.S., Texas A&M International University;

Chair of Committee: Dr. Ruby A. Ynalvez

The constant increase in environmental pollution, in particular heavy metal pollution is alarming. Cia7, the gene under study has conserved cysteine residues. These residues could be involved in metal binding; and the CIA7 protein might be associated to metal homeostasis in *Chlamydomonas reinhardtii*. The successful overexpression of Cia7 in *C. reinhardtii* could enhance its metal tolerance. The core hypothesis is that CIA7 plays a role in metal tolerance and in metal binding. The aim of this research is to characterize the Cia7 gene and to assess its potential role in metal homeostasis. The objectives of the research are (1) to overexpress CIA7 in *C. reinhardtii*, (2) to perform lead tolerance assays in WT overexpressing CIA7, and (3) to compare cell growth, chlorophyll content and bioaccumulation of heavy metals in CC5013 mutant, CC4425 wild-type and the selected transformants under the presence or absence of lead. The gene was overexpressed in wild-type (WT) and mutant (MT) *C. reinhardtii* cells. Lead tolerant colonies, the wild-type and the mutant strains were subjected to a chlorophyll content, bioaccumulation and cell growth analyses to determine if there is any difference among them. Chlorophyll content and cell growth do support the hypothesis that CIA7 could play a role in heavy metal tolerance. In the metal bioaccumulation analysis, CC4425 (WT) when compared to CC5013 (mutant), CC5013 shows higher lead bioaccumulation than CC4425. Both the parental and transformant cell consistently showed higher lead bioaccumulation, suggesting that
absence or presence of the gene did not significantly affect lead bioaccumulation. However, its absence or disruption in the mutant resulted in an increase in lead bioaccumulation. Thus, it could be inferred that Cia7 helps cells detoxify lead.

The findings of this research could provide a basis to further investigate the (1) use of Cia7 as a biomarker for metal contamination, or to (2) make the CIA7 protein be overexpressed in *C. reinhardtii* or in another organism for use in bioremediation.
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INTRODUCTION

Environmental pollution is a worldwide problem, and one of the most common pollutants is heavy metals. Industrialization is known to be one of the main factors contributing to heavy metal pollution (Fashola et al. 2016). Heavy metal pollution has become worse throughout the years; in some countries, the high levels of contamination have forced people to start taking extreme measures. For example, in China, people in highly industrialized cities have begun to use air purifiers in their houses in order to remove air pollutants (Muller et al. 2015). Another case is the city of Ulaanbaatar in Mongolia, considered one of the most air polluted cities. The pollution has reached an extreme point in the citizens' lives. In this city, oxygen cocktails are being sold and consumed by the citizens to cope with the smog presented in the air (UNICEF, 2016).

Heavy metal pollutant exposure is dangerous to humans. Human health is compromised when lead exposure is present. It could cause damage to the nervous systems, loss of hearing, and several blood related disorders (Lam et al. 2007). Therefore, there is a need to find ways to combat heavy metal pollution. Several organisms, mainly bacteria, have been studied as bioremediation organisms to cope with this problem (Ibuot et al. 2017; Arora et al. 2016; Boufadel et al. 2016; Halder et al. 2014; Kumar and Gunasundari 2018; Rezvani et al. 2018). On the other hand, the unicellular algae *Chlamydomonas reinhardtii*, the model organism for photosynthesis, is also being investigated as a bioremediation organism. *Chlamydomonas reinhardtii* copes with heavy metal pollution and has been demonstrated to have tolerance to metals (Hanikenne 2003; Aguilera and Amils 2005; Hanikenne et al. 2005; Lin et al. 2013; Osundeko et al. 2014).

*Chlamydomonas reinhardtii* has a novel gene Cia7 isolated from one of the high CO₂ requiring mutants designated as CC5013 (Ynalvez and Moroney 2008).

---

This thesis follows the model of *Environmental Toxicology and Chemistry*. 
The mutant CC5013 was generated by random insertional mutagenesis and was among the mutants that do not grow well under low CO₂ conditions (Adams et al. 2005). The CIA7 protein has 104 amino acids and by using protein-protein BLAST and domain searches, CIA7 protein was identified to have a conserved domain found in 91 hypothetical proteins from bacteria. Three are defined as zinc finger proteins and another homologous protein in *Idiomarina loihiensis*, with 78% identity to CIA7 was described as an uncharacterized conserved metal-binding protein.

The core hypothesis is: **CIA7 plays a role in metal tolerance and in metal binding.** This research aims to address whether CIA7 performs a role in *C. reinhardtii*’s tolerance to metal toxicity. Thus, to address if CIA7 performs a role in *C. reinhardtii*’s tolerance to metal toxicity, CIA7 will be overexpressed in *C. reinhardtii*. Cell growth, chlorophyll content and bioaccumulation in the CC5013 mutant, the CC4425 wild-type and selected transformants will be analyzed and compared. *Chlamydomonas reinhardtii* is a model organism for the study of metal tolerance. Lead is the heavy metal used for this research. The hypothesis are: (1) CC4425, the wild-type strain and CC5013, the mutant strain will have lower chlorophyll, cell density and biomass compared to the transformants, strains overexpressing Cia7 when exposed to sub-lethal concentration of lead. (2) There will be a difference in lead bioaccumulation among CC4425, CC5013 and the transformants. The successful overexpression of CIA7 in *C. reinhardtii* conferring enhance metal tolerance will be a basis to further test its potential for bioremediation and will provide a basis to bioengineer Cia7 into other organisms to further maximize capacity for heavy metals’ removal in either soil or water.
LITERATURE REVIEW

Environmental pollution due to heavy metals

Industrialization has been reported to be one of the main factors regarding environmental pollution. Factories have been releasing heavy metal pollutants such as Ag (Silver), As (Arsenic), Cd (Cadmium), Pb (Lead) and Hg (Mercury) to the environment for a long time. The increase and accumulation of these heavy pollutants are the consequences of an increase in mining activities and other activities influenced by humans (Fashola et al. 2016). The exploitation of natural resources carries social and economic benefits to nations, and the long term effects of heavy metals have been manifesting and increasing throughout the years. Scientists have been expecting these effects for some time (Fashola et al. 2016); thus, several studies have focused on studying the accumulation of these metals. According to Wu et al. (2014), the accumulation of these metals has been a threat for mangroves from the discharges of industrialization; this is a clear example of the threat of the heavy metal accumulation to organisms.

Pollution is one of the main problems around the world. In many countries including the United States, billions of dollars have been spent in cleaning contaminated environments and finding better methods to reduce contamination (Portney et al. 1990). Current standard cleaning processes are able to transfer the pollutants to other environments. This causes a spread of contaminants, producing risks to the close communities, which live around these contaminated places. In addition, these cleaning processes are not able to restore the environments to the proper health standards for living organisms (Yang et al. 2015; Fashola et al. 2016; Fernández-Caliani et al. 2009; Clarkson 2002). In this regard, several studies have been focusing on studying the effects of heavy metals to living organisms.
Cadmium has been investigated in order to further understand the detrimental effects in human health (Yang et al. 2015). Cadmium affects human health by mainly damaging the kidney and the bones. Metal accumulation and re-absorptive dysfunction has been found within the kidneys when cadmium poisoning is present. On the other side, demineralization of the bones has been found under cadmium exposure. Itai-Itai is considered a disease mainly caused by cadmium poisoning (Satarug et al. 2009). It was reported that the early onset of the Itai-Itai disease has a relationship to the apparent cadmium pollution of the area (Inaba et al. 2005).

Under low and high exposure, one of the most noticeable effects of cadmium is tubular impairment (International Programme on Chemical Safety 1992). The relationship between diabetes and cadmium was investigated; it was found that there is a dose response between the cadmium levels found in the urine and an increased risk of diabetes (International Programme on Chemical Safety 1992). Cadmium has been classified as a cancer causing agent in humans; and there is a high frequency of lung cancer and mortality under high cadmium exposure (Vainio et al. 1993).

Cadmium pollution is produced through different sources (Mortvedt and Osborn 1982). Volcanic activity is the main natural source of cadmium due to the production and dispersion of volcanogenic aerosols. Vegetation is another natural source, where cadmium is mainly found (Pan et al. 2010). Cadmium is found within the agriculture sector due to the application of fertilizers and the high use of sewage sludge for plants. (Panagapko 2007). These anthropogenic sources are caused by humans; other examples of anthropogenic sources are the emissions of cadmium by factories, fuel and waste incineration; also mining is considered as an activity with tons of repercussions (International Cadmium Association 2000).

Lam et al. (2007), discussed some of the effects of lead to human health; it was shown that human health is compromised by damage to the central and peripheral nervous systems, loss of hearing, several blood related disorders such as anemia, high blood pressure and decrease of hemoglobin production. Children are more vulnerable to lead poisoning than
adults. There has been an increase in growth and cognitive development retardations, behavioral problems and shortened attention span among children (Liu 2001). Lead exposure affects cognitive functioning and neurobehavioral development (Jusko et al. 2007). Another detrimental effect of the environmental pollution by lead is in innate immunity (Kasten and Lawrence 2014). Lead has a large impact on macrophages, which are part of the innate immunity. If the innate immunity is affected, it can affect several organs. Lead decreases phagocytosis and chemotaxis of macrophages, consequently, reducing the whole immune system’s capacity (Kasten and Lawrence 2014). In another study, using structural equation modeling (a statistical analysis technique that is used to analyze structural relationships, mostly used in other sciences as social and psychological sciences), it was demonstrated that lead levels affect human renal function by comparing the relationship between heavy metal burden and renal function (Trzeciakowski et al. 2014).

Environmental pollution by lead is a rising problem. Areas near mines suffer the most because active and inactive mines pile up wastes; consequently, the exposure to lead in these areas is high. In an abandoned mine in Spain, it was calculated that the surface soils in this mine have a concentration of lead over 100 times greater than undisturbed and natural soil (Fernandez-Caliani et al. 2009). On the other hand, the concentration of lead in active mines was similar to the concentration found in the mining wastes (MacKenzie and Pulford 2002). While mining is one of the most important anthropogenic sources, industrial activities or fuel use are also considered anthropogenic sources. Some natural sources include erosion or leaching, but these do not contribute as much as mining (Strmiskova 1992).

**Bioremediation as a tool for environmental cleaning**

Government agencies have supported investigations regarding pollution issues, and scientific communities have discovered new interesting methods to clean up the environment...
especially by bioremediation. Several studies have demonstrated that bioremediation could be an affordable, effective and easy way to clean-up contaminated places (National Research Council U.S., 1993). Bioremediation is described as an eco-friendly technology because it is a tool used for environmental cleaning. It is capable of removing different kind of pollutants from different environments, but specifically from soil and water. One bioremediation method is known as in situ bioremediation, which principally focuses on the treatment of contaminated places by treating them on site without creating a change to the environment (Kumar and Gunasundari 2018). This method is a better way to clean-up polluted places than the old standard methods; to overcome a lot of the issues brought by the standard cleaning methods, bioremediation has a peculiar way to clean-up polluted places such as the use of organisms as cleaning agents.

Bioremediation focuses on modifying biological agents such as bacteria, plants, fungi and algae which are microorganisms used to change the metal accumulation and toxicity within some environments. These microorganisms are principally modified in their biosynthetic pathways related to cell structure and function. As it is shown by Ibuot et al. (2017) where the transportation of metals across the membrane was overexpressed with the CrMTP4 gene. The processes in which substrates are converted into more complex products or by-products is part of the microbial metabolism. As humans need some organic compounds to live, there are some microorganisms such as bacteria which use inorganic compounds to grow and reproduce. These organic or inorganic compounds used for the growth and reproduction of organisms are known as substrates in the biosynthetic pathways (Koenigsberg et al. 2005). The organisms can use these substrates to obtain energy. Some substrates encountered in some of these pathways, if found in excessive amounts and concentrations are known to be contaminants in the environment. Therefore, by means of technology, these microorganism have been modified to be able to use these contaminants as their main source of substrate and increase their
capacity of bioaccumulation which makes them a good and effective method of diminishing pollution (National Research Council U.S. 1993).

Not only microorganisms are used for bioremediation purposes, plants and animals are used as well. Plants are able to remove contaminants from the soil; this activity is known as phytoremediation, which focuses on the absorption of contaminants into plants. Plants and earthworms can be used as organisms to remove pollutants. Plants use this common mechanism known as phytoextraction in which it involves accumulating the metals in the roots and shoots, later, these plants are collected and incinerated (Chibuike et al. 2014). In the case of animals such as earthworms, these organisms form a symbiotic relationship with bacteria where earthworms are used as an aid for the replication and activation of bacteria, and this bacteria use the contaminants as substrate (Van 2008). In some cases, organisms work as hosts or produce compost, creating a rich nutrient environment for the proliferation of bacteria (Van 2008). It was demonstrated that some microalgae species are capable of being excellent organisms for bioremediation; these algae were able to resist, and bioaccumulate heavy metals (Shanab et al. 2012). Several microalgae have been investigated, mainly fresh water microalgae. Microalgae have the capacity for heavy metal uptake, and the capacity for metal tolerance. The use of microalgae as detoxification systems is known as phycoremediation (Halder 2014).

The potential of Chlamydomonas reinhardtii as a bioremediation organism

Chlamydomonas reinhardtii was demonstrated to have a great system for detoxification against metals (Howe et al. 1992). Chlamydomonas reinhardtii strains were studied to determine their potential as organisms for bioremediation and biofuel (Hasan et al. 2014). This organism was able to survive, tolerate and bioaccumulate chemical toxicants, with a surprising rate of removal of 89.2%, 46% and 48.8% for ammonia nitrogen (Hasan et al. 2014).
Another study focused on investigating the optimal operational parameters for nutrient removal and biomass production by *C. reinhardtii*. It was demonstrated that *C. reinhardtii* exhibited a medium capacity for toxicity removal; meaning that *C. reinhardtii* has a natural good capacity for tolerance and removal of toxins which makes it an appropriate organism for bioremediation (Rezvani et al. 2018). Another study explored a genetic modification approach. The chicken class II metallothionein (MT-II) gene, was shown to enhance tolerance of the algal cells in the presence of cadmium toxic concentrations (Hua et al. 1999). The role of the heat shock protein (HSP) 70 gene was also investigated within *C. reinhardtii* in order to study its effects against heavy metals. It was found that in some cases, the tolerance and accumulation was enhanced (Schroda et al. 1999). Also metal binding was enhanced by overexpressing HISN3 (the gene coding for phosphoribosylformimino-5-aminomidazole carboxamid ribonucleotide isomerase) in *C. reinhardtii*, there was an increase in histidine concentration by 50%. The chelation of some metals happens via histidine binding, therefore nickel tolerance in *C. reinhardtii* was increased (Qi et al. 2013). Not only is histidine a way for increased metal tolerance, antioxidant activity can also be a factor. Pyrroline-5-carboxylate synthase (P5CS) is a gene that plays a key role in proline biosynthesis, leading to osmoregulation in plants. If it was overexpressed in *C. reinhardtii*, it increased metal tolerance. It is thought that the increase in metal tolerance was due to enhanced antioxidant activity (Siripornadulsil et al. 2002).

There is high potential of *C. reinhardtii* as a bioremediation organism, as it is presented by several advancements in bioremediation. Algae have been used for bioremediation before; in one study, *C. debaryana* was tested and shown to be an effective bioremediation agent. The results showed the use of *C. debaryana* to be an efficient method for the removal of wastewaters' nitrogen, total phosphorus, chemical oxygen, total organic carbon, sodium, calcium, potassium and magnesium (Arora et al. 2016). In another study, CrMTP4, a gene encoding a metal tolerance protein (MTP) in *C. reinhardtii*; was overexpressed (Ibuot et al. 2017). The tolerance of *C. reinhardtii* in different concentrations of metals was studied. It was
found to be able to tolerate magnesium (Mn) and cadmium (Cd). However in the case of Cd, the algae was not only able to tolerate but also bioaccumulate it. These results show the potential for *C. reinhardtii* to be a bioremediation organism in the future.

**Chlamydomonas reinhardtii in heavy metal homeostasis**

*Chlamydomonas reinhardtii* has been used for many studies of physiological processes such as photosynthesis, respiration, nitrogen assimilation, flagella motility and basal body function (Rochaix et al. 1998); making this organism an excellent model organism for several kinds of studies. The advances in genetics have made possible the sequencing of the whole genome of *C. reinhardtii* (Davies and Grossman, 1998). *Chlamydomonas reinhardtii* is known to resist some concentrations of heavy metals; but, at some point, heavy metals are able to cause damage to the algae. For instance, mercury decreases the growth of *C. reinhardtii* (Weiss-Magasic et al. 1997). Lead causes a decrease in the photosynthesis process, and also makes severe structural changes within the cells, specifically in the thylakoid, mitochondria and nucleus (Irmer et al. 1986). Cadmium, copper and zinc exposure decreases its nitrate uptake in the biosynthetic pathway (Devriese et al. 2001).

*Chlamydomonas reinhardtii* has high capacity for copper uptake, which was mediated by a high affinity copper transport system active in both copper-supplemented and copper-depleted conditions (Hill et al. 1996). In another study, it was reported that iron starvation leads to a rapid and large increase of cell surface, and an increase ferric-chelate reductase and ferricyanide reductase activities (Eckhard and Buckhout 1998). The response of *C. reinhardtii* to metal stress is a broad topic, many of the mechanisms under heavy metal stress are still being investigated. Heavy metal exposure inhibits the growth of *C. reinhardtii* under the exposure of different heavy metals (Jamers et al. 2009; Samadani et al. 2018). In a study by Gunawan et al. (2013), the toxicological effects of titanium and zinc oxide in *C. reinhardtii* were investigated. They
demonstrated that titanium and zinc oxide inhibits the growth of the algal cells but the inhibition does not have a correlation with reactive oxygen species (ROS) generation. They concluded that the cytotoxicity was not related to cellular oxidative stress. Structural changes have been found to be caused by heavy metal exposure as reported by Aguilera and Amils (2005) and some of the structural changes found were starch accumulation, cytoplasmic electron-dense granules, and cytoplasmic vacuolization. The development of membranous organelles and an increase of cell size were found when *C. reinhardtii* was exposed to acute concentrations and with chronic exposure to copper and cadmium (Visviki and Rachlin 1994). Oxidative stress is another consequence of the exposure of heavy metals, as some heavy metals are capable of producing reactive oxygen species (ROS) by the multiple oxidation states that some metals undergo (Stoiber et al. 2013). Perreault et al. (2011), studied the effects on *C. reinhardtii* of cadmium exposure, and inhibition of photosynthetic activity was observed.

**Chlamydomonas reinhardtii has defense mechanisms against heavy metals**

Although heavy metals may cause damage to *C. reinhardtii*, the alga has interesting defense mechanisms against heavy metal toxicity, which other organisms do not possess. The effects on *C. reinhardtii* by heavy metal exposure are controlled by different complexes (Eapen 2007). These complexes are known to be able to accumulate metals and resist its effects. It has been found that metallic cations are the first protection barrier, making *C. reinhardtii* tolerant to a certain heavy metal exposure. *Chlamydomonas reinhardtii* highly depends on its cell wall and its tolerance will be different if its cell wall is disrupted. Gekeler et al. (1989) were able to find the existence of phytochelatins (PC) in *C. reinhardtii*. These phytochelatins have been found to be metal binding peptides. The chemical reactions dealing with phytochelatins are known as PC complexes. The other mechanism of tolerance against heavy metals exposure is the regulation of gene expression in *C. reinhardtii*. 
*Chlamydomonas reinhardtii* serves as a valuable organism in the study of heavy metal homeostasis (Hanikenne 2003). Metal transporters are important for heavy metal homeostasis (Hill et al. 1996). Under cadmium exposure, *C. reinhardtii* has a mitochondrial ABC transporter, which has been found to be part of heavy metal homeostasis (Hanikenne et al. 2005). Differential gene expression is also linked to heavy metal homeostasis. Mutants have been identified to have a phenotype in metal deficiency, therefore, several metal regulators have been discovered i.e. one of the copper regulators (Crr1) (Quinn et al. 1999). A multicopper ferroxidase gene (Fox1) has been identified, which has been linked to metal homeostasis due that is involved into high affinity iron uptake (Herbik et al. 2002). MicroRNAs have also been found to be linked to heavy metal homeostasis under cadmium and copper exposure (Ding and Zhu 2009).

Another mechanism that makes *C. reinhardtii* a suitable organism under heavy metal exposure is the high affinity that the cell wall has for metallic cations (Collard and Matagne 1990). In addition, *C. reinhardtii* has metal binding peptides called phytochelatins, which function as intracellular metal chelators under cadmium exposure (Gekeler et al. 1989; Howe & Merchant 1992; Hu et al. 2001). On the other hand, in a study where *C. reinhardtii* was exposed to mercury; it was reported that *C. reinhardtii* produces thioredoxins (TRXs), proteins that play a role in oxidative stress. These proteins were found to contribute to heavy metal detoxification by binding to the reactive oxygen species, or by repairing other damaged proteins, but this is still inconclusive (Lemaire et al. 2002).

Some *C. reinhardtii* mutants were generated by insertional mutagenesis, these were reported not only sensitive to copper or cadmium, but also sensitive to lead exposure (Hanikenne et al. 2001). The deleted arg7 gene from insertional mutagenesis was linked to have heavy metal homeostasis under cadmium, copper or lead (Hanikenne et al. 2001). All these heavy metal defense mechanisms support the use of *C. reinhardtii* as an organism in the study of heavy metal homeostasis and tolerance.
EXPERIMENTAL SECTION

Cell culturing and maintenance

*Chlamydomonas reinhardtii* strains CC4425 wild type and CC5013 mutant (Cia7 deletion) were purchased from the *Chlamydomonas* Resource Center, University of Minnesota. Cell cultures (cells) were maintained at room temperature (≈22°C) with an overhead fluorescent light source (250 µmol photons/m²/s). Cells were maintained as stocks in 1.5% TAP (Tris-acetate-phosphate) media, stored at room temperature and re-plated every week. The transformants were maintained in TAP-paromomycin plates. Liquid cultures, where by one to two loopfuls of cells were used to inoculate 50 mL TAP media. Cell cultures were grown up to log phase. An absorbance of 0.8, OD₆₅₀ was calibrated to be equal to 2.8 x 10⁶ cells/mL (log phase in *C. reinhardtii*) (Shin et al. 2017). Absorbance readings were taken using a Spectronic Genesys 8 spectrophotometer©. Culture flasks were placed in an orbital shaker (VWR DS2-500-1) at 130 rpm.

Plasmid isolation and purification

*Escherichia coli* clones with a) the plasmid designated as pSL72-Cia7 (plasmid with the Cia7 insert) previously generated in our laboratory and b) plasmid pSL72 were used (Gonzalez-Cantu et al. 2017). The bacterial clones were inoculated in 1-5 mL LB medium containing the selective antibiotic ampicillin. The cell cultures were incubated for 12-16 hours at 37 °C, harvested and centrifuged at 8000 rpm for 3 minutes at room temperature. The plasmid with Cia7 insertion had a size of 6230 bp, and with no Cia7 had a size of 5495 bp. These were isolated and purified using Qiagen’s©© Mini QIAprep Spin Miniprep Kit (Cat. No. 27104) following the manufacturer’s instructions.
**Restriction enzyme digestion and gel electrophoresis**

To verify that the plasmid had the insert (Cia7 gene), restriction enzyme digests were performed. Restriction enzymes NotI (R0189S) and BamHI (R0136S) were used. Restriction enzyme digests were done following the manufacturer’s protocol (New England Biolabs). NotI (R0189S) and BamHI (R0136S) were chosen as these enzymes allowed differentiation in fragment sizes between a clone without the insert and the clone with the insert. Restriction enzyme NotI (R0189S) was used to linearize the plasmids used in the eukaryotic transformations. NotI (R0189S) was chosen because this enzyme cut only at the multiple cloning site and not at any region of the plasmid, thus it did not affect any critical gene within the plasmids. The DNA concentration from the plasmid was measured using a spectrophotometer (Thermo Fisher Scientific – NanoDrop-2000©). Analysis of DNA fragments were done using DNA gel electrophoresis with a 0.8% agarose gel.

**Eukaryotic transformation of C. reinhardtii**

Eukaryotic transformation was carried out by electroporation, following the methodology by Ynalvez (2007). Wild type (CC4425) and the mutant strains (CC5013) were grown in 100 mL of TAP media until log phase (OD$_{650}$=0.8). When cells reached the log phase, the cells were transferred to 1 L of TAP. After 24 hours cells were harvested, centrifuged at 3500 rpm, and pellets were resuspended to a cell density of 2 x10$^8$ cells mL$^{-1}$ in Tap + 60mM sorbitol. One μg of Not I- linearized psL72-Cia7 was added to 250 μL of cells in a 0.4 cm electroporation cuvette (Bio-Rad – cat no. 1652081). The mixture was incubated 15 minutes in ice. This was then subjected to a capacitance of 25 μF, 800 V with no shunt resistor (∞) using the Bio Rad Gene Pulser Xcell™. Pulse time averaged between 11-14 ms. The cells were allowed to recover in 10 mL TAP + 60 mM sorbitol overnight. Cells were harvested by centrifugation for 1 minute at 3,500 rpm, and resuspended with the remaining TAP-sorbitol after decanting
supernatant. Cells with a density of approximately $6 \times 10^7$ cells/mL were plated onto TAP plates with paromomycin (5 µg/mL final concentration).

The vector, pSL72, used in this experiment has a strong upregulating promoter psaD. It is the promoter for the nuclear gene psaD which encodes an abundant protein of the photosystem I reaction center subunit II in photosynthetic organisms. The psaD promoter is used to drive efficient gene expression in *C. reinhardtii* (Fischer and Rochaix 2001). This vector also has the selective marker AphVIII (shown in Figure 1). This marker confers 5 µg/mL paromomycin resistance. Therefore, transformants were selected based on their growth in plates containing paromomycin. Transformants were maintained on TAP-paromomycin plates and were re-plated every two weeks.

![Figure 1](image.png)

**FIGURE 1.** Vector map of linearized pSL72-Cia7. This was the plasmid used to overexpress Cia7 in *C. reinhardtii*. Relevant restriction sites are shown.

**Screening and preliminary characterization of CC4425 and CC5013 transformants**

The transformants were resistant to paromomycin, as conferred by the AphVIII gene. In order to determine if Cia7 overexpressing transformants will have an increased Pb$^{2+}$ tolerance, metal tolerance assays were performed. The transformants were resuspended in minimal media
and 5 µL of this suspension was pipetted onto Min + Pb(NO$_3$)$_2$ plates (lead concentrations: 0 М, 750 М and 1500 М). The cells were grown for approximately 5-7 days and observed for phenotypic differences. The hypothesis is: overexpressing Cia7 in CC4425 and CC5013 will result in higher tolerance to lead as shown by a healthier phenotype than the parental strain, a healthier phenotype is demonstrated by a darker green color intensity. Score “3” with the darkest green color show the healthiest phenotype as opposed to score “1” with the pale green color (Figure 2) when subjected to Pb$^{2+}$ stress.

**FIGURE 2.** Color chart to be used to assess healthy or less healthy phenotype when compared to the wild-type strain. (1) – (3) left to right: (1) was assigned to colonies that are light green; (2) was assigned to colonies less green; (3) was assigned to dark green colonies.

**Chlorophyll analysis, cell density, cell count and biomass determination**

Heavy metals have been documented to inhibit microalgae growth (Miazek et al. 2015; Carfagna et al. 2013; Nowicka et al. 2016; Weiss-Magasic et al. 1997). Chlorophyll damage and synthesis inhibition to microalgae is another consequence of heavy metal contamination. Measurements of chlorophyll content have been reported as a useful physiological tool to assess the extent of heavy metal damage (Bakar et al. 2015). Cell growth monitoring was done by cell density measurements, cell count, and biomass determination.

Cells or strains namely: CC4425, CC5013, and the transformants were grown on TAP media until an absorbance of 0.8, OD$_{650}$. This absorbance reading has been calibrated to be equal to 2.8 x $10^6$ cells/mL and equal to C. reinhardtii’s log phase (Shin et al. 2017). The cells were harvested and pelleted using a centrifuge at a 4200 rpm for 10 minutes. The pellets were
washed with minimal media and 250 mL of culture with Min media of 0.5 OD$_{650}$ was subjected to Pb treatment (0 µM, 100 µM). Samples were collected at time intervals of 0, 24, 48 and 72 hours.

**Cell density and chlorophyll analysis:** For each trial, one mL of culture from each treatment was collected for the cell density and the chlorophyll content. Cell density was monitored with spectrophotometric measurement at OD$_{650}$. Chlorophyll content was obtained by the acetone extraction method (Schagerl et al. 2007). The cell sample was centrifuged at 4200 rpm for 10 minutes, and the supernatant was removed. The pellet was resuspended with 1 mL of 80% acetone. This suspension was centrifuged, and the supernatant was transferred into a cuvette. The absorbance was read at 663 nm and 645 nm to determine chlorophyll A, B, and total chlorophyll content (Schagerl et al. 2007).

**Cell count:** One mL of cells was collected and 111 µL of iodine was added into the sample and transferred into a Neubauer chamber. The cells were analyzed under a compound microscope (Yang et al. 2017; Wink et al. 2014; Chioccioli et al. 2014).

**Biomass determination:** For each trial, a 50 mL centrifuge tube was pre-weighed; and fifty mL of cell suspensions were collected and then centrifuged. The supernatant was removed, and the cell pellets were dried at 85 °C overnight. The dried cells were weighed and re-dried until no more change in weight was observed. The difference between the pre-weight and the post weight was recorded as the biomass (Berberoglu et al. 2008).

To analyze the data, an analysis of variance associated with a 5 x 1 x 2 x 4 factorial experiment in randomized complete block design was performed. The factorial arrangement of treatments was the result of five levels of strain (CC5013, CC4425, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72), one level of metal (Pb), 2 levels of metal concentration (0 µM, 100 µM) and 4 levels of time (0, 24, 48, and 72 hours). To compare significant main and interaction effects, post-hoc test in the form of comparing least squares means was performed using PROC GLM of the SAS 9.4 statistical software. To further explore
the nature of significant interaction between metal and concentration, a t-test comparing concentration levels was performed for each media level. The usual levels of type-I error rates were used (i.e., * if p < .05, ** if p < .01 and *** if p < .001).

**TABLE 1.** Treatment scheme for cell growth and chlorophyll analysis of CC4425 and CC5013 per replication for CC4445 (WT), CC5013 (cia7 mutant) and transformants: CC4425 +pSL72-Cia7 (vector plus insert), CC5013 +pSL72-Cia7 (vector plus insert) and CC4425 +pSL72 (vector only).

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<th>24 hour</th>
<th>48 hour</th>
<th>72 hour</th>
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**Bioaccumulation analysis using ICP-OES**

Measurements of intracellular metal accumulation have been reported as indicators of metal tolerance (Fernandez and Novillo 1995; Pérez-Rama et al. 2002; Collard and Matagne 1994). *Chlamydomonas reinhardtii* cells were cultured in liquid media on an orbital shaker (VWR) until an OD$_{650}$ of 0.8 +/- 0.05, using a Spectronic Genesys 8® spectrophotometer. Preliminary results from experiments from our laboratory had established a sub-lethal lead concentration of 100 µM for strains CC4425 and CC5013. Cells were cultured in acetate minimal media (Min media) treated with Pb(NO$_3$)$_2$ at concentrations of 0 µM and 100 µM to a final volume of 150 mL. Sample collections were done at 0, 3, 6, 9 hours, and at 24, 48 and 72 hours.
TABLE 2. Bioaccumulation treatment scheme per replication for CC445 (WT), CC5013 (cia7 mutant) and transformants: CC4425 +pSL72-Cia7 (vector plus insert), CC5013 +pSL72-Cia7(vector plus insert) and CC4425 +pSL72 (vector only).

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<tr>
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<td>CC4425 +pSL72 (vector only)</td>
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Treatment flasks were washed with 1% HNO₃ to prevent metal cross-contamination among replicates. At each collection time, 3 mL of cells per trial were equilibrated at 0.8 ABS₆₅₀. This is an absorbance which gave an equal number of cells for all treatments and also enough for all the analyses. The cells were pelleted by centrifugation at 3,000 rpm for 10 minutes and were washed with deionized water and resuspended and re-pelleted by centrifugation at 3,000 rpm for 10 minutes. Pellets were desiccated at 100°C and were either frozen at -80°C or used for downstream analysis. Dry pellets were acidified with 3 mL of HNO₃, incubated for 20 minutes, and digested in a MARS 6® microwave following machine’s internal protocol for plant material. Following digestion, samples were diluted to a factor of 1:20, and analyzed for Pb.
utilizing a Varian Agilent series ICP-OES® (Inductively coupled plasma – optical emission spectrometer). Three replications were conducted with 3 trials per replication.

An analysis of variance associated with a 5 x 1 x 2 x 4 factorial experiment in randomized complete block design was performed. The factorial arrangement of treatments was the result of five levels of strain (CC5013, CC4425, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72), one level of metal (Pb), 2 levels of metal concentration (0 µM, 100 µM) and 4 levels of time (0, 24, 48, and 72 hours). To compare significant main and interaction effects, a post-hoc test in the form of comparing least squares means was performed using PROC GLM of the SAS 9.4® statistical software. To further explore the nature of significant interaction between metal and concentration, a t-test comparing concentration levels was performed for each media level. The usual levels of type-I error rates were used (i.e., * if p < .05, ** if p<.01 and *** if p<.001).
RESULTS AND DISCUSSIONS

Eukaryotic Transformation of CC4425 and CC5013

Although, the plasmid with pSL72-Cia7 was isolated from a clone previously generated in our lab, the plasmid was verified prior to use in the transformation experiment. If the Cia7 insert is present in plasmid, pSL72-Cia7, the expected size for each band according to the enzyme cut sites will be 5,662 bp and 568 bp with a total size of 6230 bp. Results showed the presence of the insert in the plasmid pSL72-Cia7 (Figure 2). Therefore, the plasmid isolated from the identified clones were used in electroporation. However, confirmation of the presence of the insert by sequencing was not done and is recognized as a limitation and weakness of this experiment.

FIGURE 3. Verification of pSL72-Cia7 plasmid by restriction enzyme digestion with NotI and BamHI. The gel displays two bands, 1st band with a size of 5662 bp, and 2nd band with a size of 568. Lane 1: MW ladder. Lane 2-7: pSL72-Cia7 digested in two bands. Lane 8: Negative control (molecular grade water).

The transformants generated from CC4425 (WT) were 500 colonies (Figure 4) with only one transformation experiment, and only 33 colonies from 3 transformations were generated
from CC5013 (MT) (Figure 5A). There was higher transformation efficiency observed in the WT strains compared to the mutant strains. The mutant strains could be less resistant to the electroporation conditions compared to the WT. Under normal conditions, the growth rate of CC5013 is slower when compared to CC4425. Therefore, when CC5013 was exposed to stressful conditions, the strain was not able to sustain much stress from transformation conditions. In addition, higher plasmid concentration was used for the CC5013 (3 μg compared to 1 μg used for the CC4425) transformation. The plasmid pSL72 (vector only) was used as a control in this experiment resulting in 12 transformants generated from a single transformation (Figure 5B).

**FIGURE 4.** Growth media plates with 5 μg/mL of paromomycin (A-D) showing the WT (CC4425) cells transformed with pSL72-Cia7. CC4425 did not grow in any of these plates.

**FIGURE 5.** Growth media plate with 5 μg/mL of paromomycin showing (A) the mutant strain (CC5013) transformed with pSL72-Cia7. (B) CC4425 transformed with pSL72 (Vector only control) Parental strains, CC5013 nor CC4425 did not grow in these plates.
Cloning approaches were reported as an effective method for studies in gene characterization in algae (Ermilova et al. 2000; Van and Clijsters 1990; Hanikenne 2003; Ferrante et al. 2008; Lin et al. 2013). Gene overexpression has been a great way to study metal tolerance in *C. reinhardtii*. For example, Wei et al. (2011) overexpressed the HO-1 (Heme oxygenase-1 gene) and the transformants showed metal tolerance when exposed to Hg (mercury). There was a 48.2% increase of cell count but a decrease in metal accumulation when compared to the wild type. Wang et al. (2015) overexpressed the CrGNAT gene and showed Cu (Copper) metal tolerance in *C. reinhardtii* transformants. In a study conducted by Ibuot et al. (2017), researchers focused on characterizing the function of CrMTP4. CrMTP4 was reported to be responsible for sequestering Cd (Cadmium) and other metals. This gene has potential for bioremediation due to its broad capacity to sequester a variety of metals. Previous studies have demonstrated the importance and the efficacy of overexpressing genes as a way to characterize a variety of genes in *C. reinhardtii*. Thus, the transformants successfully generated from eukaryotic transformation in this present study were used for further analysis.

**Determination of Pb sub-lethal concentration in CC4425 and CC5013**

The lead sub-lethal concentration was determined for the metal tolerance assay. The metal tolerance assay qualitatively assessed phenotypic differences among CC4425 (WT), CC5013 (MT) and the transformants (parental strains +Cia7) when subjected to lead stress. Thus, in this experiment, the objective was to find a lead concentration whereby cells, CC4425 and CC5013 will be less healthy (assessed qualitatively by color) but cells would still survive. In this regard, in the metal tolerance assay, transformants more metal tolerant than the parental strains, CC4425 and CC5013 were identified. Cells, CC4425 (WT) and CC5013 (mutant) were exposed to different lead concentrations, 0 - 2 mM were tested. The sub-lethal concentration was established at 1.5 mM of Pb. At this concentration, cell colonies showed a phenotypic difference compared to the colonies exposed to zero lead concentration (Figure 6).
FIGURE 6. Lead Sub-Lethal Assay was performed to establish a concentration allowing for the observation of phenotype difference of CC4425 and CC5013 between lead treatment and no treatment. (A) under no lead treatment (B) at 1.5 mM Pb treatment.

The lead sub-lethal assay is essential for the metal tolerance assay. Other studies have used different approaches, but they all fall into the same paradigm. As explained by Beauvais-Flück et al. (2016), the sub-lethal concentration was found by using different methylmercury concentrations that would affect C. reinhardtii. Another study in which sub-lethal toxic concentration of copper and cadmium were found was done by Xie et al. (2019). Xie et al research’s focus was to predict the sub-lethal effects of pollutants by developing a DEBtox model. On the other hand, Hanikenne et al. (2001) found cadmium, copper, lead and mercury sub-lethal concentrations for Chlamydomonas spp. transformants by using plates with different concentrations of metals. This assay was similar to what was done in this study. This assay was useful to find colonies which have higher resistance to lead compared to the parental strains when exposed to 1.5 mM Pb(NO₃)₂.
**Metal tolerance screening of CC4425 and CC5013 transformants**

A qualitative approach was done prior to the quantitative approach of characterizing the transformants. A spot method was employed, whereby cells were diluted with liquid minimal media and 15 µl for each colony were pipetted into the plate. Results showed colonies transformed with plasmid pSL72 + Cia7 that had better metal tolerance compared to parental strains (Figure 7).

![FIGURE 7](image1.png)

*FIGURE 7.* Results from the metal tolerance screening assay. A phenotypic difference, whereby 8.8% of the transformants (CC4425+Cia7) showed metal tolerance at 1.5 mM of lead compared to the WT CC4425 (indicated by arrows); parental strain. WT CC4425 did not survive in 1.5 mM lead.

Out of the 500 colonies obtained from *C. reinhardtii* CC4425 transformation, 44 colonies showed higher lead tolerance than WT. Three replications were carried out and results were consistent across 3 replications (Figure 8). On the other hand, out of the 33 colonies obtained from *C. reinhardtii* CC5013 transformation, only 7 transformants showed the same or even higher metal tolerance in comparison to the parent strain, CC5013 at 1.5 mM lead (Figure 9).

A semi-quantitative assessment was done for the CC4425+Cia7 transformants that survived under 1.5 mM lead exposure (Figure 10). The assessment was done to compare the cell growth of each of the transformants with those of the parental strain. It was also used as a reference to choose the transformants for further analyses.
FIGURE 8. Third replication of 44 transformants (CC4425+Cia7) that showed higher metal tolerance in 0 mM lead and 1.5 mM lead. A and C; as well as B and D represent trials 1 and 2, respectively.

FIGURE 9. Representative plates from the metal tolerance screening assay of the 12 mutant transformants (CC5013) in 0 mM lead and 1.5 mM lead. A and C; B and D represent trials 1 and 2, respectively.
FIGURE 10. Semi-quantitative assessment of CC4425 transformants. The colonies were “scored” according to the colony color’s intensity. “1” (Light green), “2” (Less green), “3” (Dark green).

Colonies that scored "1" were 43.7% ± 17.55%, while colonies that scored “2” were 38.24% ± 10.08%, and colonies that scored “3” dark green even under 1.5 mM lead were 18.1% ± 13.5%. Again, a phenotypic difference, whereby 8.8% (44) of the 500 transformants (CC4425+Cia7) showed metal tolerance at 1.5 mM of lead compared to the WT CC4425.

Preliminary chlorophyll analysis of CC4425 transformants

The purpose of this experiment was to compare the cell growth and total chlorophyll content of the transformants with the parent strain, CC4425. Cells, CC4425 (parent strain) and CC4425 plus insert (the transformants) at midlog phase (OD650=0.4) were exposed to 100 μM lead concentration and were analyzed after 72 hours.
**FIGURE 11.** Total chlorophyll content after 72 hours exposure to 100 μM lead. Strain 1 is CC4425 (Wild Type) and Strain 2-54 are transformants (CC4425+pSL72-Cia7)

**FIGURE 12.** Cell density after 72 hours exposure to 100 μM lead. Strain 1 is CC4425 (Wild Type) and Strain 2-54 are transformants (CC4425+pSL72-Cia7)
Total chlorophyll content of the 53 transformants (CC4425+pSL72-Cia7) were compared with CC4425 (wild type) (Figure 11). The control, CC4425 did not show a difference in total chlorophyll content between 0 and 100 μM. However, 10 transformants (18.86%) showed significant difference in total chlorophyll content between 0 and 100 μM lead. On other hand, in Figure 12, 53 transformants (CC4425+pSL72-Cia7) were compared to CC4425 under metal and nonmetal exposure. CC4425, the control did not show significant difference in cell density between 0 and 100 μM lead exposure. It was observed that 14 transformants (26.4%) showed significant difference in cell density between 0 and 100 μM lead exposure. These results indicated that there were transformants (CC4425+pSL72-Cia7) generated in the transformation experiment that have potential to have metal tolerance; as evidenced by higher total chlorophyll and cell density even when exposed to high lead concentration of 100 μM.

**Chlorophyll content analysis under Pb exposure among different strains (CC4425, CC5013, CC4425+Cia7 and CC5013+Cia7) at different time intervals**

Detrimental effects caused by metal exposure in *C. reinhardtii* can be assessed through gene expression analysis, transcripts, or oxidative metabolites (detection of reactive oxygen species through a fluorescence microscope at 600 ms) as seen in Flück et al. (2016); Wang et al. (2015); Ibuot et al. (2017) and Wei et al. (2011). Other researchers used hybridization procedures such as the southern blot analysis (Hanikenne et al. 2001; McHugh et al. 1994). The analysis most common in these studies (Xie et al. 2019; Beauvais-Flück et al. 2016; Ibuot et al. 2017; Wang et al. 2015) was chlorophyll content analysis. Chlorophyll damage and synthesis inhibition in microalgae are consequences of heavy metal contamination (Irmer et al. 1986). Measurements of chlorophyll content have been reported as a useful physiological tool to assess the extent of heavy metal damage (Bakar et al. 2015).
Three colonies out of the 7 metal-tolerant colonies (based on higher total chlorophyll content compared to WT), 14 metal-tolerant colonies (based on higher cell density compared to WT) and 1 colony out of the 6 colonies from the CC5013 (MT) transformation were chosen for this experiment. The strains were analyzed under 100 uM lead exposure at different time intervals 0, 24, 48 and 72 hours. The strains were: 1) CC4425 (WT), 2) CC5013 (MT), 3) CC4425+pSL72 (control), 4) CC4425+pSL72-Cia7 and 5) CC5013+pSL72-Cia7. There were three different colonies used from CC4425+pSL72-Cia7, these colonies were also labeled as T1, T2 and T3. It has been reported that lead can cause deleterious damage to the metabolism and physiology of microalgae (Bakar et al. 2015), in this regard the objective of this experiment was to determine and compare the chlorophyll content among these 5 strains upon exposure to lead.

**TABLE 3.** Analysis of variance results for Chlorophyll A. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the time and the strain variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>6</td>
<td>289.428433</td>
<td>48.2380722</td>
<td>4.29</td>
<td>0.0039</td>
</tr>
<tr>
<td>Metal</td>
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<td>0.1045405</td>
<td>0.1045405</td>
<td>0.01</td>
<td>0.9239</td>
</tr>
<tr>
<td>Strain*Metal</td>
<td>6</td>
<td>37.1112726</td>
<td>6.1852121</td>
<td>0.55</td>
<td>0.765</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>22.8691279</td>
<td>7.6230426</td>
<td>7.68</td>
<td>0.0001</td>
</tr>
<tr>
<td>Strain*Time</td>
<td>18</td>
<td>48.2578888</td>
<td>2.6809938</td>
<td>2.7</td>
<td>0.0012</td>
</tr>
<tr>
<td>Metal*Time</td>
<td>3</td>
<td>2.9882146</td>
<td>0.9960715</td>
<td>1</td>
<td>0.3957</td>
</tr>
<tr>
<td>Strain<em>Metal</em>Time</td>
<td>18</td>
<td>20.2733034</td>
<td>1.1262946</td>
<td>1.13</td>
<td>0.3355</td>
</tr>
</tbody>
</table>

R-Square = .897337; Coefficient of Variation = 18.48846.
FIGURE 13. Chlorophyll A at 0, 24, 48, and 72 hours. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for chlorophyll A. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.

TABLE 4. Analysis of variance results for Chlorophyll B. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the time and the strain variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Type III SS</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>Strain</td>
<td>6</td>
<td>31.5875973</td>
<td>5.26459955</td>
<td>2.24</td>
<td>0.0711</td>
</tr>
<tr>
<td>Metal</td>
<td>1</td>
<td>0.40719905</td>
<td>0.40719905</td>
<td>0.17</td>
<td>0.6807</td>
</tr>
<tr>
<td>Strain*Metal</td>
<td>6</td>
<td>8.10163199</td>
<td>1.350272</td>
<td>0.57</td>
<td>0.7471</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>9.73576145</td>
<td>3.24525382</td>
<td>7.88</td>
<td>0.0001</td>
</tr>
<tr>
<td>Strain*Time</td>
<td>18</td>
<td>31.3176855</td>
<td>1.73987142</td>
<td>4.23</td>
<td>0.0001</td>
</tr>
<tr>
<td>Metal*Time</td>
<td>3</td>
<td>1.27915385</td>
<td>0.42638462</td>
<td>1.04</td>
<td>0.3812</td>
</tr>
<tr>
<td>Strain<em>Metal</em>Time</td>
<td>18</td>
<td>7.72418028</td>
<td>0.42912113</td>
<td>1.04</td>
<td>0.424</td>
</tr>
</tbody>
</table>

R-Square = 0.816561, Coefficient of Variation = 25.48492
FIGURE 14. Chlorophyll B at 0, 24, 48, and 72 hours. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for chlorophyll B. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.

TABLE 5. Analysis of variance results for total chlorophyll. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the time and the strain variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
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</thead>
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<td>Strain</td>
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<td>504.2243252</td>
<td>84.0373875</td>
<td>3.62</td>
<td>0.0096</td>
</tr>
<tr>
<td>Metal</td>
<td>1</td>
<td>0.095992</td>
<td>0.095992</td>
<td>0</td>
<td>0.9492</td>
</tr>
<tr>
<td>Strain*Metal</td>
<td>6</td>
<td>75.9445158</td>
<td>12.6574193</td>
<td>0.55</td>
<td>0.7688</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>61.8318424</td>
<td>20.6106141</td>
<td>8.64</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Strain*Time</td>
<td>18</td>
<td>137.8982738</td>
<td>7.6610152</td>
<td>3.21</td>
<td>0.0002</td>
</tr>
<tr>
<td>Metal*Time</td>
<td>3</td>
<td>7.0296968</td>
<td>2.3432323</td>
<td>0.98</td>
<td>0.4054</td>
</tr>
<tr>
<td>Strain<em>Metal</em>Time</td>
<td>18</td>
<td>47.9436441</td>
<td>2.6635358</td>
<td>1.12</td>
<td>0.3521</td>
</tr>
</tbody>
</table>

R-Square = .879989; Coefficient of Variation = 19.54071.
FIGURE 15. Total Chlorophyll Content across 0, 24, 48, and 72 hours. The data above indicates that there was significant difference through the four different time intervals. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for total chlorophyll content. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.

Using $\alpha = 0.05$, there was no significant difference in chlorophyll A, chlorophyll B and total chlorophyll content among parental strains and transformants. However, there was significant difference in chlorophyll A, chlorophyll B and total chlorophyll content at 72 hours in T3-CC4425+pSL72-Cia7 and CC5013+pSL72-Cia7 (Tables 5-7; Figures 12-14).

*Chlamydomonas reinhardtii*’s chlorophyll A, chlorophyll B and total chlorophyll increased as time increased.
Metal tolerance in *C. reinhardtii* has been previously reported, whereby chlorophyll content was used as a marker. In a study conducted by Wei et al. (2011), *C. reinhardtii* was transformed with the heme oxygenase-1 gene. The chlorophyll content was found to be significantly different among the transformants (control and transgenic *C. reinhardtii*) and the metal treatment (control and mercury treatment). Heme oxygenase-1 gene was reported to contribute to metal homeostasis (Wei et al. 2011). In a different study, Ibuot et al. 2017 found metal tolerance among different metal treatments (aluminum, copper and zinc) and among different strains (*Chlamydomonas reinhardtii, Chlorella luteoviridis, Parachlorella hussii*, and *Parachlorella kessleri*); the overexpressed gene designated as CrMTP4 was reported to confer metal tolerance.

Results in tables 3, 4 and 5 indicated no statistically significant difference among strains and metal treatments (p>0.05). The results showed that insertion of the Cia7 gene did not have an effect on the chlorophyll content of the cells when exposed to lead. However, it was observed at 72 hours, that chlorophyll A, chlorophyll B and total chlorophyll content showed significant difference in T3-CC4425+pSL72-Cia7 and CC5013+pSL72-Cia7 in comparison to the rest of the strains (p<0.05) (Figure 13-15). Chlorophyll content tends to increase at 48 hours and decrease at 72 hours (Figure 13-15). For chlorophyll A, chlorophyll B, and total chlorophyll content; T3-CC4425+Cia7 and CC5013+Cia7 had the significantly lowest values at 72 hours (Figure 13-15). These results are interesting because the two strains which showed the significant difference were transformants with Cia7’s insertion. According to the results (Figures 13-15), Cia7 seems to be affecting the chlorophyll synthesis of these strains. However, this is not conclusive and still needs further investigations.

**Cell density, biomass determination and cell count under Pb exposure among different strains (CC4425, CC5013, CC4425+Cia7 and CC5013+Cia7) at different time intervals**

Aquatic microorganisms such as microalgae are very sensitive to the toxic effects of metals; metal exposure has shown to inhibit cell growth (Awad and Chu 2005). Cao et al. (2015)
demonstrated the effects of lead in the green alga _Cladophora_. It was shown how at certain lead concentration, lead has detrimental effects over the organism, causing a decrease of cell growth, and an increase of intracellular metal accumulation. The detrimental effects on microalgae under cadmium and copper have also been determined (Jamers et al. 2013; Kumar and Gaur 2014). Inhibition of cell growth is an indicator of microalgae damage under metal exposure. In these analyses, it was hypothesized that transformants: CC4425+Cia7 and CC5013+Cia7 which were overexpressing Cia7 had an increase in cell growth compared to parental strains CC4425 and CC5013. Cell growth was analyzed by determination of cell density. This analysis is one of the most common and a simple method to determine cell growth (Kumar and Gaur 20t14). However, spectrophotometric analysis cannot differentiate between living or dead cells, therefore, cell count was also performed. Since biomass determination is another way to analyze cell growth, biomass determination was used to complement the other two methods.

**TABLE 6.** Analysis of variance results for cell density. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the time and the strain variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
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<td>Strain</td>
<td>6</td>
<td>0.88568578</td>
<td>0.1476143</td>
<td>4.29</td>
<td>0.0039</td>
</tr>
<tr>
<td>Metal</td>
<td>1</td>
<td>0.01217883</td>
<td>0.01217883</td>
<td>0.35</td>
<td>0.5569</td>
</tr>
<tr>
<td>Strain*Metal</td>
<td>6</td>
<td>0.06369544</td>
<td>0.01061591</td>
<td>0.31</td>
<td>0.9266</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>0.10985045</td>
<td>0.03661682</td>
<td>7.17</td>
<td>0.0002</td>
</tr>
<tr>
<td>Strain*Time</td>
<td>18</td>
<td>0.20668585</td>
<td>0.01148255</td>
<td>2.25</td>
<td>0.007</td>
</tr>
<tr>
<td>Metal*Time</td>
<td>3</td>
<td>0.00605083</td>
<td>0.00201694</td>
<td>0.4</td>
<td>0.7569</td>
</tr>
<tr>
<td>Strain<em>Metal</em>Time</td>
<td>18</td>
<td>0.04676912</td>
<td>0.00259828</td>
<td>0.51</td>
<td>0.947</td>
</tr>
</tbody>
</table>

R-square = .847219; Coefficient of Variation = 14.45303.

There was no significant difference among the strains tested. Lead exposure did not significantly affect cell growth. Cell density was not significantly different among strains. Using \( \alpha = 0.05 \), there was a significant difference in cell density at 0 hours for CC4425 when compared to the rest of the strains (\( p<0.05 \)). Across 24, 48 and 72 hours; there was significant difference
in cell density for T3-CC4425+pSL72-Cia7 and CC5013+pSL72-Cia7 in comparison to the rest but T2-CC4425+pSL72-Cia7 (p<0.05). As time increased, cell density increased.

**FIGURE 16.** Cell density at 0, 24, 48, and 72 hours. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for cell density. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.

**TABLE 7.** Analysis of variance results for cell count. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the time and the strain variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>6</td>
<td>566506.4038</td>
<td>94417.734</td>
<td>11.39</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Metal</td>
<td>1</td>
<td>1093.4386</td>
<td>1093.4386</td>
<td>0.13</td>
<td>0.7193</td>
</tr>
<tr>
<td>Strain*Metal</td>
<td>6</td>
<td>8096.8353</td>
<td>1349.4725</td>
<td>0.16</td>
<td>0.9844</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>18477.5435</td>
<td>6159.1812</td>
<td>3.12</td>
<td>0.0302</td>
</tr>
<tr>
<td>Strain*Time</td>
<td>18</td>
<td>121335.121</td>
<td>6740.8401</td>
<td>3.42</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Metal*Time</td>
<td>3</td>
<td>2895.0875</td>
<td>965.0292</td>
<td>0.49</td>
<td>0.6906</td>
</tr>
<tr>
<td>Strain<em>Metal</em>Time</td>
<td>18</td>
<td>27630.9143</td>
<td>1535.0508</td>
<td>0.78</td>
<td>0.7189</td>
</tr>
</tbody>
</table>

R-square = .854023; Coefficient of variation = 12.4458.
Using $\alpha = 0.05$, there was a significant increase in cell count for the strains over time. Thus, as time increased, *C. reinhardtii*'s cell count differed among the strains. The highest value at 72 hours was 479.98 +/- 407.88 cells in T3-CC4425+pSL72-Cia7, however, this was not significantly different compared to the other time intervals ($p>0.05$). Cell count significant difference was found at 48 hours with CC4425 ($p<0.05$). T3-CC4425+pSL72-Cia7 and CC4425+pSL72 were significantly different at 72 hours when compared to the rest of the strains but CC5013+pSL72-Cia7 ($p<0.05$).

**FIGURE 17.** Cell count across 0, 24, 48, and 72 hours. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for total cell count. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.
TABLE 8. Analysis of variance results for biomass determination. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the time and the strain variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
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<th>Pr &gt; F</th>
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<tbody>
<tr>
<td>Strain</td>
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<td>0.01013775</td>
<td>0.00168963</td>
<td>8.43</td>
<td>&lt;.0001</td>
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<tr>
<td>Metal</td>
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<td>0.00110744</td>
<td>0.00110744</td>
<td>5.52</td>
<td>0.0266</td>
</tr>
<tr>
<td>Strain*Metal</td>
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<td>0.00079938</td>
<td>0.00013323</td>
<td>0.66</td>
<td>0.6786</td>
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<tr>
<td>Time</td>
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<td>0.00041274</td>
<td>0.00041274</td>
<td>3.28</td>
<td>0.0811</td>
</tr>
<tr>
<td>Strain*Time</td>
<td>6</td>
<td>0.00396513</td>
<td>0.00066085</td>
<td>5.24</td>
<td>0.001</td>
</tr>
<tr>
<td>Metal*Time</td>
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<td>0.00003407</td>
<td>0.00003407</td>
<td>0.27</td>
<td>0.6071</td>
</tr>
<tr>
<td>Strain<em>Metal</em>Time</td>
<td>6</td>
<td>0.00062802</td>
<td>0.00010467</td>
<td>0.83</td>
<td>0.5563</td>
</tr>
</tbody>
</table>

R-square = .864572; Coefficient of variation = 28.65017.

FIGURE 18. Biomass Determination across 0 and 72 hours. The data above indicates that there was significant difference between the two-time intervals. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for biomass determination. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.
Using $\alpha = 0.05$, there was significant difference of biomass for strains over time. It can be observed that after 72 hours, CC5013 showed the most significant difference in biomass when compared to the other strains (Figure 18).

Cell growth (cell density, cell count and biomass determination) is used to analyze the detrimental effect of heavy metal exposure. There was an increase of cell growth after 4 days of metal exposure in cells overexpressing CrMTP4 (Ibuot et al. 2017) and cell growth after 8 days of metal exposure in cells overexpressing CrGNAT (Wang et al. 2015) compared to the parental strain. According to the results of cell growth in this study, the strains were not significantly different among each other under metal exposure, but these were significantly different across the different time intervals (Tables 8-10) (Figures 15-17). This shows that even under metal exposure, cell growth was not affected under the presence or absence of lead. Interestingly, at 72 hours, CC5013 (mutant) showed significant difference in biomass with 0.079 +/- .061 g (p<.05) (Figure 17). The strains, with CC4425 background (WT and transformants) had lower biomass (p<.05).

Although CC5013+pSL72-Cia7 was the third highest for biomass determination (.052 +/- .033) at 72 hours, it had significant different biomass determination in comparison to CC5013 (0.079 +/- .061) at 72 hours (Figure 18). According to these results, Cia7 may be affecting the overall cell growth of the strains but in an opposite manner than what was expected. Most heavy metals, including lead, have been shown to decrease cell growth (Arora et al. 2016). While many of these studies are not related to Cia7 or the strains used for this study, these investigations have shown lead’s potential to cause changes in the structure and morphology of microalgal cells. The results of this experiment indicate that lead did not contribute to significant changes in cell growth among the strains. This observation suggests that there is no correlation between the Cia7 gene’s function and the metal conditions tested in this study. Based on these results, it is unlikely that Cia7 contributes to metal homeostasis. However, Cia7 is significantly
affecting the cell growth among the strains, so further research must be done. For future studies, the metal tolerant transformants could be used for further analysis and sources of variations will have to be examined thoroughly.

**Determination and comparison of Pb bioaccumulation among different strains (CC4425, CC5013, CC4425+Cia7 and CC5013+Cia7) at different time intervals**

Intracellular metal accumulation has shown to be a great indicator for metal tolerance in *C. reinhardtii* (Fernandez and Novillo 1995; Pérez-Rama et al. 2002; Collard and Matagne 1994). The mechanism for *C. reinhardtii* to deal with heavy metal exposure is to absorb the metal intracellularly. Once metal concentration reaches a certain threshold, cellular uptake is performed through various mechanisms such as endocytosis or metal protein transporter as explained by Brautigam et al. (2011). Since CIA7 was hypothesized to be a protein involved in metal tolerance in *C. reinhardtii*, the overexpression of this protein might have an effect on metal bioaccumulation in the strains with the Cia7 insert (transformants). Therefore, the purpose of this analysis is to determine and compare the metal bioaccumulation among the parental strains and the transformants. The strains used were CC4425, CC5013, and the transformants (CC4425+pSL72-Cia7 and CC5013+pSL72-Cia7). The hypothesis is that overexpressed CIA7 strains will have higher or lower lead bioaccumulation levels when compared to the parental strains (CC4425 and CC5013).

**TABLE 9.** Analysis of variance results for lead bioaccumulation. Among the different variables tested (Strains, Metal treatments and Time), it is shown how the strain and the metal treatment variables are significant different among each other.

<table>
<thead>
<tr>
<th>Source</th>
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<th>F Value</th>
<th>Pr &gt; F</th>
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</thead>
<tbody>
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<td>Strain</td>
<td>6</td>
<td>339201.3635</td>
<td>56533.5606</td>
<td>5.59</td>
<td>0.0008</td>
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<tr>
<td>Metal</td>
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<td>925482.5881</td>
<td>925482.5881</td>
<td>91.47</td>
<td>&lt;.0001</td>
</tr>
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<td>Strain*Metal</td>
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<tr>
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<td>7349.9353</td>
<td>1.24</td>
<td>0.1859</td>
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R-square = .705246; Coefficient of variation = 204.8778.
FIGURE 19. Bioaccumulation across the two metal treatments in different strains. Strains CC4425, CC5013, CC4425+pSL72-Cia7, CC5013+pSL72-Cia7 and CC4425+pSL72 were analyzed for metal bioaccumulation. During this analysis, there were three different colonies used for strain CC4425+pSL72-Cia7, these were labeled as T1, T2 and T3. In total there were seven different transformants or colonies tested.

Results indicate that there was significant difference in the two metal treatments across the different strains. Bioaccumulation increased when exposed to lead indicating metal exposure affects all C. reinhardtii strains including transformants with Cia7 insertion. CC5013+pSL72-Cia7 showed the highest bioaccumulation (259.95169.71) when exposed to 100 uM (p<.002). The strain with the lowest bioaccumulation was found to be CC4425 (46.82 ppm 43.41). At 0 uM lead, no significant statistical difference was observed among strains. However, as expected there was significant difference between the two metal treatments (p<0.0001). There were significant differences among strains at 100 μM.
The highest bioaccumulation was CC5013+pSL72-Cia7’s as 259.95 +/- 169.71, followed by T3’s as 194.53 +/- 104.29, T2’s as 140.71 +/- 50.47, CC4425+pSL72’s as 137.36 +/- 47.12, T1’s as 102.22 +/- 11.98, CC5013’s as 90.26 +/- .026, and CC4425’s as 46.82 ppm +/- -43.41. CC5013+pSL72-Cia7 was not significantly different (p>0.05) compared to T3, but it was significantly different from the parental strains.

Bioaccumulation is another analysis that relates to metal tolerance genes. Beauvais-Flück et al. (2016) and Xie et al. (2019) reported that bioaccumulation depends mostly in the algae’s mechanism for tolerating metals. Therefore, depending on the gene’s function, algae would bioaccumulate certain heavy metals at greater levels over others, or some genes might contribute to the increase or decrease the bioaccumulation of the metal (Wang et al. 2015; Ibuot et al. 2017 and Wei et al. 2011). Wang et al. (2015) and Ibuot et al. (2017) reported that metal bioaccumulation was increased in the transgenic cells compared to the wild type. In these investigations the function of the genes is believed to be related with histone methylation or oxidative stress, respectively. On the other hand, Wei et al. (2011) showed a decrease in metal accumulation, a result likely due to the possible function behind the studied gene. According to the author, it is hypothesized that this gene increases antioxidative capacity which improves the integrity of the cell membrane and most likely prevents Hg from loading into the cells.

The insertion of Cia7 resulted in an increase of metal bioaccumulation for T2, T3 and CC5013+pSL72 (Figure 19). In Figure 19, four out of the five transformants showed significant difference in comparison to the wild type (CC4425) under 100 µM lead exposure. These results suggest that Cia7 could increase the capacity of metal accumulation. An increase in metal accumulation from previous studies was attributed to histone methylation or oxidative stress (Wang et al. 2015; Ibuot et al. 2017). CC5013+pSL72-Cia7 showed higher bioaccumulation than T3-CC4425+pSL72-Cia7. This is of interest because if CC4425 (WT) is compared to CC5013 (mutant), CC5013 shows higher lead bioaccumulation than CC4425. Both the parental
and transformant cells consistently showed higher lead bioaccumulation, suggesting that the absence or presence of the gene did not significantly affect lead bioaccumulation. However, its absence or disruption in the mutant resulted in an increase in lead bioaccumulation. Thus, it could be inferred that Cia7 helps cells detoxify lead.
CONCLUSIONS AND RECOMMENDATIONS

Microalgae have evolved to cope with the damaging effects of heavy metal exposure (Howe et al. 1992). These organisms are capable of sequestering metals and removing them before causing detrimental effect in the cell’s physiology (Hasan et al. 2014). Other mechanisms that microalgae use to cope with metal toxicity include histone methylation and regulation of reactive oxygen species states (ROS) (Wang et al. 2015; Ibuot et al. 2017). Since environmental pollution is an ongoing environmental problem, interpreting and making use of these metal mechanisms could be of relevance for developing solutions to deal with heavy environmental pollution. Since the CIA7 protein has conserved cysteine-rich motifs, this protein is hypothesized to play a role in heavy metal tolerance. This study successfully achieved the possible overexpression of CIA7 in CC4425 (WT) and CC5013 (mutants) strains.

Even though transformation for CC4425 was a success, CC5013’s transformation still needs improvement. When Pb sub-lethal concentration was determined in CC4425 and CC5013, it was of interest because CC5013 (Mutant) with no Cia7 showed lower metal resistance compared to CC4425 (Wild type). Nevertheless, it was more interesting that some transformants were more tolerant than CC4425 when exposed to 1.5 mM lead. Cia7 insertion might be increasing metal tolerance in the transformants with Cia7 insertion.

During the preliminary chlorophyll analysis of CC4425 transformants, there were transformants that had higher chlorophyll content even when exposed to lead. On the other hand, additional experiments did not show significant difference in cell growth among strains in the absence or presence of lead. In the metal bioaccumulation analysis, CC4425 (WT) when compared to CC5013 (mutant), CC5013 showed higher lead bioaccumulation than CC4425. Both the parental and transformant cells consistently showed higher lead bioaccumulation, suggesting that absence or presence of the gene did not significantly affect lead bioaccumulation. However, its absence or disruption in the mutant resulted in an increase in
lead bioaccumulation. Thus, it could be inferred that Cia7 helps cells in heavy metal homeostasis.

The results of this investigation where Cia7 insertion showed higher metal tolerance in comparison to the parental strains provide a basis to keep further investigating CIA7 as a possible biomarker for metal contamination in the future, gene expression could be quantitatively analyzed to address the expression of CIA7. Further studies must be done to approach CIA7 as an overexpressed protein for bioremediation purposes, in any organism.
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Research:

Ethanolamine in *Morganella Morganii* (01/24/2018-2018)

- Due to the lack of information of *Morganella Morganii*, this research was dedicated to understand and investigate the different pathways and genes that are present in this opportunistic bacteria. Ethanolamine has been found in several microorganisms, and it is thought that ethanolamine can be a main compound for the pathogenesis of this bacteria.

Characterization of *Chlamydomonas reinhardtii* CIA7 under heavy metal conditions (08/25/2018-2020)

- CIA7 is a novel gene discovered in *Chlamydomonas reinhardtii*, its function is still unknown. It seems to have a vital function involving heavy metal accumulation, this research studies this gene under heavy metal conditions as it could be a potential gene for bioremediation.

The typist for this thesis was Mr. Juan Carlos Onofre Renteria.