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**A promoter trap screen to discover genes induced by metals in  
*Chlamydomonas reinhardtii***

**Dana Simon**

**A Thesis**

**in**

**The Department**

**of**

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## ABSTRACT

A promoter trap screen to discover genes induced by metals in  
*Chlamydomonas reinhardtii*

Dana Simon

This thesis initiated a long term project that uses the green alga *Chlamydomonas reinhardtii* to identify new mechanisms of defence to toxic trace metals and pathways that regulate these responses. Strains of *C. reinhardtii* that express reporter genes under the control of alga's metal response were generated and analysed. The reporter gene, an *Arg7* cDNA minigene, restores arginine prototrophy following its insertion into random sites of the *C. reinhardtii* genome where it is expressed from resident regulatory sequences. Cells were selected and screened by for rare insertions that express the reporter gene only in the presence of bioavailable metal. For all eight transformants induction of the *arg*<sup>+</sup> phenotype is not specific to particular metal ions. Southern analysis showed that two strains, Ni70 and Cd3600, have only one insertion of the reporter gene. Northern analysis showed that Ni70 and Cd3600 activate expression of the introduced *Arg7* cDNA, at the RNA level by nickel and cadmium. Initial results, from a real time RT-PCR assay on strain Ni70, demonstrated that increasing concentrations of nickel or cadmium results in an increase of the reporter gene expression. Therefore, Ni70 indeed appears to have the reporter gene inserted under the influence of promoter / enhancer which is activated by metal exposure.

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## LIST OF ABBREVIATIONS

apoPCs:	phytochelatin peptide without metal ion
ABC:	ATP binding cassette
CRE:	cAMP response element
EDTA:	Ethylene diaminetetraacetic acid
FIAM:	Free ion activity model
GSH:	reduced glutathione
GS-X:	Glutathione S-conjugated
MRP:	Multidrug resistance proteins
MTs:	Metallothioneins
PC:	Phytochelatins
ROX:	dark red dye (maximum emission at 595 nm)
RT-PCR:	Reverse transcription polymerase chain reaction
SDS:	Sodium dodecyl sulphate
SYBR:	SYBR Green dye (maximum emission at 520 nm)
TAP:	Tris-acetate-phosphate growth media
Tris:	Tris(hydroxymethyl) aminomethane
TRX:	Thioredoxin

## CHAPTER 1. INTRODUCTION

### 1.1 Stress responses

As a consequence of industry and human activity there is an enormous and increasing release of toxic trace metals that leads to their high accumulation in the biosphere. These heavy metals stay largely in the aquatic and soil phases of the planet and adversely affect all organisms (Zenk, 1996). Examples of heavy metals include mercury, cadmium, arsenic, chromium, thallium, and lead. Heavy metals are the most important pollutants, but others metals (e.g. nickel) are also relevant pollutants and it is valuable to understand how organisms sense and respond to them in order to mitigate their toxic effects. In plants, exposure to trace metals results in reduced rates of photosynthesis, chlorosis, growth inhibition, decrease in water and nutrient uptake, and death (Siripornadusil *et al.*, 2002). In humans and animals, exposure to trace metals can induce cancer, kidney damage and other health disorders (Desi *et al.*, 1998). Understanding how microorganisms tolerate toxic concentrations of metals can also provide insight into strategies for their detoxification or removal from the environment (Bruins *et al.*, 2000). Moreover, it is of fundamental interest to understand how organisms tolerate adverse conditions, and how they regulate these responses. The effects of metal toxicity, especially cadmium (Cd) and nickel (Ni), will be focused on as well as the defense mechanisms characterized to date in eukaryotes.

## 1.2 Metal toxicity

The mechanisms of metal toxicity are not fully understood and many remain to be identified. Metal toxicity depends on metal speciation and concentration. Many metals serve essential biological roles, e.g. as cofactors for a wide range of biochemical reactions. However, all metals are toxic above a certain concentration of bioavailable species. Metals are found in different forms, chemical species, including free hydrated ions, complexed with natural ligands or adsorbed on the surface of particles and colloids. Metal speciation is therefore determined by multiple factors such as water hardness, pH, organic molecules and colloids (Tessier and Turner, 1995). The speciation of a metal affects its ability to be taken up by cells and to interact with cellular components. As a consequence, different species of a metal vary greatly in toxicity. The free ion activity model (FIAM), proposes that toxicity is related to the concentration of the divalent metal cation. The chelated metal is less bioavailable and therefore less toxic to the organism. For example, the relationship between the lead speciation and its bioaccumulation by the green alga *Chlorella vulgaris* was studied and short term uptake experiments demonstrate the biouptake was governed by the free lead ion activity (Slaveykova and Wilkinson, 2002).

Metal toxicity is determined by concentration and necessity in the cell. As mentioned before, some metals such as cobalt, copper, iron and zinc are essential. They serve as micronutrients, are used as cofactors for catalysis of redox reactions and stabilize molecules through electrostatic interactions (Zhu and Thiele, 1996). When their concentration is too low the organisms activate tightly controlled

mechanisms for essential metal ion uptake. For example, in the unicellular green alga, *Chlamydomonas reinhardtii*, a copper uptake pathway has been identified with high affinity for copper ions when they are deficient (Hill *et al.*, 1996). Other metals, which are nonessential (such as Cd, silver, lead, beryllium, chromium, arsenic and mercury) are toxic to the organism even in picomolar quantities (Ahner *et al.*, 1994). It had been commonly believed that essential metal ions were in equilibrium with metalloproteins. However, at least in *Saccharomyces cerevisiae*, the free copper concentration is far less than a single atom per cell; the vast majority of copper ions are bound to chelators and chaperones (Rae *et al.*, 1999).

Metals exert toxicity through a variety of mechanisms. Metals undergo aberrant reactions with the thiol groups of proteins, promote the formation of active oxygen species, alter enzyme specificity, and displace endogenous metal cofactors. Examples of each are given below.

Many biosynthetic enzymes are inhibited due to interaction of metal ions with their thiol groups. For example, in *Hordeum vulgare*, protochlorophyllide reductase is highly sensitive to cadmium interaction with SH-groups normally involved in the binding of NADPH (Stobart *et al.*, 1985). Cadmium also binds *in vitro* to SH-groups involved in stabilization of ribulose-bisphosphate carboxylase-oxygenase (Rubisco) from barley leaves, causing dissociation of subunits and loss of activity (Stiborova *et al.*, 1986). In *C. reinhardtii*, a cadmium-mediated increase in reactive oxygen species leads to a decrease in the reduced/oxidized

glutathione ratio, which in turn signals the translational arrest of the Rubisco large subunit (Irihimovitch and Shapira, 2000).

Trace metals can also displace endogenous metal cofactors and  $\text{Ca}^{2+}$  from signaling proteins and enzymes. For example, yeast Cu, Zn-superoxide dismutase is inhibited by silver through binding of silver to the copper-binding site (Ciriolo *et al.*, 1994).

Metals also promote the formation of active oxygen species, which damage cell membranes and DNA. Nickel is a potent carcinogen and can induce malignant transformation of rodent and human cells (Salnikow *et al.*, 1999). Cadmium enhances the mutagenicity and carcinogenicity of directly acting genotoxic agents by inhibiting DNA repair (Beyersmann and Hechtenberg, 1997). Thus, at high levels, both essential and nonessential heavy metals are toxic. Thus, the cell has developed several defense mechanisms against metal toxicity.

### **1.3 Cell defenses against metals**

Organisms adapt to the toxic effects of metals with a variety of tolerance mechanisms such as: (1) binding or sequestration by metal-complexing agents, (2) transport of metals to a subcellular compartment (e.g., to vacuoles), (3) extracellular sequestration (4) excretion (Rauser, 1995, Robinson *et al.*, 1993) and (5) metal transformation. Microbial activity is responsible for the transformation of at least one third of the elements in the periodic table. These transformations are the result of assimilatory, dissimilatory, or detoxification processes (Stolz *et al.*, 2002). Excretion of metals is a system mostly used by prokaryotic organisms

(Nies, 1992). Some organisms use a combination of these systems to protect themselves against the damage. This introduction will focus in the first three mechanisms.

Metal detoxification within cells is mediated by binding or sequestration by metal-complexing agents, such as phytochelatins (PC). PCs are  $(\gamma\text{-Glu-Cys})_n\text{-Gly}$  metal thiolate polypeptides. They are atypical in that they are non-translationally synthesized. Rather, they are synthesized from glutathione by phytochelatin synthase ( $\gamma$ -glutamylcysteine dipeptidyl transpeptidase). PCs bind metal ions via their thiol groups (Vatamaniuk *et al.*, 2000, Rauser, 1995). Phytochelatin synthase has been found in plants, yeast and algae (Vatamaniuk *et al.*, 1999, Al-Lahham *et al.*, 1999, Klapheck *et al.*, 1994, Kubota *et al.*, 2000). Among the toxic heavy metals, Cd is the strongest inducer of phytochelatin synthesis. *Rauvolfia serpentina* cells exposed to 100  $\mu\text{M}$  Cd produced 30 times more PC than when exposed to 100  $\mu\text{M}$  Ni (Steffens, 1990). Interestingly, the diatom *Thalassiosira weissflogii* induces phytochelatin synthesis when the concentration of free cadmium is as low as 1 pM (Ahner *et al.*, 1994). It must be noted that researchers debate whether nickel induces the production of phytochelatins. In 1985, it was reported that nickel induces production of phytochelatins in *R. serpentina* (Grill, 1985) while a later study affirmed that phytochelatin production was not detected in the presence of nickel (Zenk 1996). In *C. reinhardtii*, cadmium induces production of phytochelatins, as was detected by HPLC. The same study revealed that silver does not induce at significant



levels and mercury was primarily bound to glutathione peptides, not phytochelatin (Howe and Merchant, 1992).

Metallothioneins (MTs) are cysteine-rich gene-encoded polypeptides which sequester metals. While it was believed that MTs were present only in animals, recent reports demonstrate that they are also present in plants (Liu, 2002). The completion of the *Arabidopsis thaliana* genome sequence has allowed the identification of the entire suite of MT genes in this vascular plant (Cobbet, 2002). Metallothioneins have not been found in *C. reinhardtii* but they are present in the marine alga *Fucus vesiculosus* (Morris, 1999).

Low molecular weight organic molecules, mainly organic acids (citrate, malate or malonate), amino acids and their derivatives (Andrew, 1995) also play important roles in plant metal homeostasis. Proline ameliorates environmental stress in plants and microorganisms, including heavy metal stress. Expression of a mothbean delta(1)-pyrroline-5-carboxylate synthetase (P5CS) gene in *C. reinhardtii* results in 80% higher free proline levels, relative to wild-type cells. These cells grow more rapidly in presence of toxic cadmium concentrations (100  $\mu$ M) and bind four-fold more Cd than wild-type cells. The results suggest that the free proline acts as an antioxidant in Cd-stressed cells and that higher glutathione levels facilitate increased phytochelatin synthesis (Siripornadulsil *et al.*, 2002). In addition, in hyperaccumulator plants (metal accumulating plants, able to grow in soils rich in metals), elevated production of histidine is more important than sequestration of toxic metals by organic acids (Andrew, 1995).

In plants, sequestration of the chelated metal into vacuoles is another important mechanism of detoxification and tolerance. Studies indicate that metal hyperaccumulator plants can tolerate higher amounts of metal than other vascular plants, not because their production of intracellular binding molecules is higher, but because they have an enhanced compartmentalization of metal in the vacuoles (Chardonnens *et al.*, 1999, Pence *et al.*, 2000, Neumann and Nieden, 2001). A mechanism in plant cells was proposed according to which metal ions enter through the permeable cell wall, are chelated by metal binding peptides and are then transported into vacuoles via specialized transporters. In the vacuole the metal is chelated by organic acids and maintained as this chelated nontoxic form (Zenk, 1996).

Detoxification might be achieved by extracellular sequestration. The cell wall of *C. reinhardtii* is rich in hydroxyproline glycoproteins and this could provide another means of protection against metal toxicity; the ions are trapped in the negatively charged sites (Macfie *et al.*, 2000, Sumper *et al.*, 1998).

Arbuscular mycorrhizal symbioses can increase tolerance of some plants to heavy metals and alleviate stress by influencing the metal bioavailability. The mechanisms of this phenomenon are poorly understood (Hall, 2002).

#### **1.4 Metal transporters**

Nonessential metal ions, such as cadmium, are most likely transported across plant plasma membranes via transporters for essential divalent cations (e.g.  $\text{Ca}^{2+}$  transporters) and glutathione S-conjugate pumps. Expression of *LCT1*

cDNA, which encodes a putative plant cation transporter for  $\text{Cd}^{2+}$ , renders the growth of *S. cerevisiae* more sensitive to cadmium uptake and this uptake can be blocked by  $\text{La}^{3+}$  and  $\text{Ca}^{2+}$ . *LCT1* complements a yeast disruption mutant gene encoding a membrane  $\text{Ca}^{2+}$  influx system and may contribute to transport of the toxic metal  $\text{Cd}^{2+}$  ions across plant membranes (Clemens *et al.*, 1998). In *A. thaliana*, *Nramp* genes code for proteins that transport both the metal nutrient, iron, and the toxic metal, cadmium (Thomine *et al.*, 2000). In oat roots cadmium is transported into the vacuole via a  $\text{Cd}^{2+}/\text{H}^+$  antiporter using the pH gradient generated by the V-type ATPase complex and hydrolysis of ATP (Salt and Wagner, 1993). Similarly, in *Schizosaccharomyces pombe*, vacuolar  $\text{Cd}^{2+}$  sequestration is mediated by a vacuolar ATP binding cassette transporter, named heavy metal tolerance factor1 (HMT1), that catalyzes the  $\text{Mg}^{2+}$  /ATP energized uptake of Cd-PCs and apoPCs into the vacuoles of wild type but not *hmt1<sup>-</sup>* cells (Ortiz *et al.*, 1992; Ortiz *et al.*, 1995). In *S. cerevisiae* the transport of nickel into vacuoles is a pH gradient-driven process, and the pH gradient is generated by a proton pump (Nishimura *et al.*, 1998). In *C. reinhardtii* silver-thiosulfate complexes are transported across the plasma membrane via sulfate/thiosulfate transport systems and sulfate acts as a competitive inhibitor of this uptake mechanism (Fortin *et al.*, 2001). Thus, it is evident that many different metal ion transporters, using different mechanisms, can transport toxic metal ions into the cell.

Glutathione S-conjugate pumps (GS-X) represent a class of transporters called Multidrug Resistance Proteins (MRP), a subfamily of the ATP binding

cassette (ABC) transporter proteins. GS-X transporters are involved in the vacuolar sequestration or plasma membrane extrusion and detoxification of both endogenous and exogenous toxins in yeast and plants by removing the toxins from the cytosol. Substances transported by these membrane proteins are herbicides and heavy metals, such as cadmium and arsenic. Transport is selective for GS-conjugates and oxidized glutathione, but not reduced glutathione. In *A. thaliana* a transporter responsible for removal of glutathione S-conjugates from the cytosol is encoded by *AtMRP1* gene (Lu *et al.*, 1997)

Chelated toxic ions appear to be removed from the cytosol of eukaryotic organisms' by sequestration in the vacuoles. ATP binding cassette (ABC) pump proteins are being found to function in an increasing variety of uptake and efflux functions, including nutrient transport, protein and peptide transport, polysaccharide transport and ion transport. A regulated network of metal transport, chelation and sequestration activities function to provide the distribution and detoxification of metal ions. Regulation of gene responses to metal ions is necessary to understand this network.

### **1.5 Regulation of metal ion-induced gene expression**

Exposure of cells to toxic levels of free metal ions is known to stimulate the expression of various types of genes. Some of these genes encode transcriptional regulatory proteins, others are genes activated in the oxidative stress response and others are activated due to the stress induced by the metal toxicity. Examples of genes products that act as signals for other genes in metal

homeostasis are the AP1 regulatory protein, the ACE1 transcription activator protein and glutathione peptides. More general systems, such as the reactive oxidative species (ROS) system, are also activated in some organisms.

In yeast, *YAP1* encodes a transcriptional regulatory protein. Changes in the gene dosage of this factor can dramatically alter the ability of a cell to tolerate a host of toxic agents including cadmium, cycloheximide, and sulfometuron methyl. Cells deficient for *YAP1* are hypersensitive to cadmium (Wemmie *et al.*, 1994). However, genes regulated by Yap1p that are involved in resistance to toxic agents have not been studied yet.

Ace1p is a transcriptional activator protein responsible for the induction of yeast detoxification genes in response to copper. These genes include metallothionein genes (*CUP1* and *CRS5*) and *SOD1*, which encodes Cu, Zn superoxide dismutase. Both *in vivo* and *in vitro* footprinting analyses have demonstrated that Cu-activated Ace1p binds to metalloregulatory transcription factors on the *CUP1*, *SOD1* and *CRS5* gene promoters. Deletion of the *ACE1* gene renders the yeast strain extremely sensitive to copper (Marjorette *et al.*, 1998, Gralla *et al.*, 1991).

Of the many roles ascribed to glutathione (GSH) the one most clearly established is its role in the protection of higher eukaryotes against oxygen toxicity through destruction of thiol-reactive oxygen byproducts. A normal ratio of 500:1 reduced versus oxidized GSH is maintained in the cell. The binding of reduced GSH molecules to free  $\text{Cd}^{2+}$  represents a heavy drain of these molecules within the cell and leads to induction of the GSH synthesizing enzymes

(Rüegsegger *et al.*, 1990, Rüegsegger and Brunold, 1992). Overexpression of genes involved in PC synthesis, such as gamma-glutamylcysteine synthetase (*GSH1*), glutathione synthetase (*GSH2*) and phytochelatin synthase have been shown to increase cadmium tolerance in various heterologous expression systems.

In 1999, three groups simultaneously and independently cloned and characterized genes encoding phytochelatin synthase. These were isolated from *A. thaliana* (*AtPCS1*), *S. pombe* (*SpPCS1*) and wheat (*TaPCS1*) (Clemens *et al.*, 1999, Ha *et al.*, 1999, Vatamaniuk *et al.*, 1999). It has been proposed that phytochelatin synthase is activated directly by the binding of  $Cd^{2+}$  to phytochelatin synthase (Zenk, 1996, Cobbet, 1999). However, *in vitro* experiments with immunopurified recombinant *AtPCS1* demonstrated that the enzyme is only slightly activated by direct interaction with free metal ions and the primary activator is GSH-derived thiol peptides containing blocked thiol groups (Vatamaniuk *et al.*, 2000). A differential display study in which *C. reinhardtii* was exposed to 25  $\mu$ M cadmium revealed that a number of nuclear and chloroplast gene transcripts were up-regulated. The greatest induction was found for a gene with significant homology to *HCRI*, a high  $CO_2$  and iron deficiency-inducible gene from *C. littorale*. The functions of other genes induced by cadmium are related to the responses which follow various kinds of metabolic damage (Rubinelli *et al.*, 2002).

Peroxisomes respond to cadmium toxicity by increasing the activity of the antioxidative enzymes involved in the ascorbate-glutathione cycle and the NADP-dependent dehydrogenases located in these organelles. It has been shown that

toxic cadmium levels induce imbalances in the activated oxygen metabolism of pea leaf peroxisomes, but the main effect is an enhancement of the H<sub>2</sub>O<sub>2</sub> concentration of these organelles (Romero-Puertas *et al.*, 1999). The *C. reinhardtii* gene homologous to glutathione peroxidase (*Gpxh*) is up-regulated under oxidative stress conditions. *Gpxh* showed a remarkably strong and rapid induction by the singlet oxygen-generating photosensitizers neutral red, methylene blue and rose Bengal. In contrast, the *Gpxh* mRNA levels were only weakly induced by exposure to the superoxide-generating compound paraquat and to hydrogen peroxide. The *Gpxh* promoter contains a region between 104 and 179 bp upstream of the transcription start site that is responsible for the mRNA up-regulation upon exposure to singlet oxygen. Within this region a regulatory sequence homologous to the mammalian cAMP response element (CRE) and activator protein 1 (AP-1) binding site was identified within a 16 bp palindrome. (Leisinger *et al.*, 2001).

## **1.6 Methods for detecting differentially regulated genes**

The identification of genes that are differentially regulated in response to specific elicitor or environmental conditions can reveal gene products and regulatory systems involved in the response to that condition. Methods used to identify differentially regulated genes are differential display, proteomics (2D gels), microarrays and promoter trapping. Differential display techniques (Matz M.V. and Lukyanov, 1998) reveal cDNAs representing mRNAs that are present in a particular cell type and absent from a control cell type. An enrichment step

involves hybridization of cDNA representing mRNAs from a test condition to an excess of cDNAs obtained from a control mRNA population. The unhybridized fraction is then separated from the hybridized common sequences by hydroxylapatite chromatography, avidin-biotin binding or using oligo(dT)30-latex beads. Despite the successful identification of numerous important genes, such as the T-cell receptors, by these methods they are usually inefficient for obtaining low abundance transcripts. The subtraction techniques often require greater than 20 ug of poly-(A)<sup>+</sup> RNA, involve multiple or repeated subtraction steps and are labor intensive. RNA fingerprinting by arbitrary primed PCR is potentially a faster technique for identifying differentially expressed genes (Menke U. and Mueller-Roeber B., 2001). However, both of these methods reveal a high level of false positives, biased for high copy number mRNAs and might be inappropriate in experiments in which only a few genes are expected to show differential expression.

Microarrays using cDNA substrates provide a means to assess genome-wide expression patterns after exposure of an organism to different xenobiotics. Potential uses for this technology include identification of unknown toxicants, assessment of toxicity of new compounds and characterization of the cellular mechanisms of toxicant action. However, this method is expensive and requires a great deal of expertise.

At the core of proteomics is two-dimensional polyacrylamide protein gel electrophoresis (2D-PAGE) (Steven *et al.*, 2000). This technique is capable of resolving thousands of proteins and peptides from a single complex mixture in a



single experiment. Proteins are first separated according to their isoelectric point (the pH at which their net charge is zero) and then orthogonally separated based on apparent mass using an SDS-PAGE step. The individual proteins are revealed as isolated spots on the gel by applying standard staining protocols. A lot of protein species can be separated, detected and quantified in a single operation. Hundreds of the detected proteins can be identified in a highly automated fashion by sequence analysis of the peptide mixtures generated by digestion of individual gel spots. However, some classes of proteins are under-represented in 2D gel patterns. These include very acidic or basic proteins, excessively large or small proteins and membrane proteins.

A very simple low-cost procedure for mass screening of differentially regulated genes is the promoter/enhancer trap screen. Enhancer and promoter traps use a reporter gene lacking a promoter such that the gene's expression in a transformant requires its insertion into a position of the genome where its transcription is driven by a promoter. Transformant strains are screened for rare insertion events that put the reporter gene under the control of regulatory elements that respond to the condition of interest (e.g. metal toxicity). When inserted into a random site of a genome by transformation, the reporter gene is expressed only when it lies close to (enhancer trap) or within (promoter trap) another gene. The expression of the reporter gene in a transformant depends on the transcriptional regulatory elements of the gene near or into which the reporter gene has inserted. The efficiency of promoter trapping techniques for gene tagging and isolation is correlated to the frequency at which it is possible to detect reporter gene

expression. Promoter trapping has proven to be very useful for the identification of genes inducible by specific physiological or environmental factors or expressed in specific tissues or developmental stages. One advantage of this approach includes the isolation of genes encoding low abundance mRNAs. Thus, genes that are expressed in a very limited way, such as being restricted to certain cell types or being active during a very short period of development can be detected. Furthermore, this is a low cost method and does not require expensive equipment.

Few promoter trap systems currently exist in *C. reinhardtii*. GFP (Franklin *et al.*, 2002), Renilla luciferase (Minko *et al.*, 1999) and aminoglycoside adenine transferase, which confers resistance to streptomycin antibiotics (*aadA*) (Goldschmidt, 1991), reporter genes for the *C. reinhardtii* chloroplast have been designed. However, these systems do not work very well because of the codon bias in this organism which causes heterologous genes to be efficiently silenced.

### **1.7 Promoter structure in *C. reinhardtii***

Eukaryotic promoters may contain a TATA box, upstream and downstream elements, and initiators. Promoters for highly specialized genes tend to have a TATA box while promoters for housekeeping genes tend to lack them. Most genes have some kind of upstream element and many have more than one. Studies on promoters of *C. reinhardtii* tend to indicate that expression of genes requires the action of multiple different sequence elements. The promoter of the  $\alpha$ -1 Tubulin gene requires the region upstream of -56 for the gene's induction, while deletion of the region from -176 to -122 decreased the gene's induction by

half (Periz and Keller, 1997). Promoter studies of the nitrate reductase gene (*Nial*) of *C. reinhardtii* demonstrate that expression is promoted by at least four elements between -231 and -8 and suggest that part of the repression by ammonium ion takes place through a proximal element located in the sequence from -51 to -33 (Loppes and Radoux, 2001). Some enhancers are situated within introns. Promoters for nuclear genes in *C. reinhardtii* contain regulatory elements and enhancers within introns as well as in the flanking regions. In the *C. reinhardtii* dynein gene (*IC70*), which is required for flagellar regeneration, the element mediating the response to deflagellation is situated in the 5'-untranslated region (Kang and Mitchell, 1998). Other genes such as *PsaD*, which is expressed at high levels, contain no introns and the regulatory sequences required for its high-level expression lie in the flanking promoter and untranslated regions (Ficher and Rochaix, 2001). Thus, promoter structures tend to be diverse, rendering the task of identifying regulatory sequence elements difficult. To date, only a few of these sequence elements have been identified.

### **1.8 Promoter trap in *C. reinhardtii* for heavy metals**

The present study is an attempt to generate strains of *C. reinhardtii* that express reporter genes under the alga's metal response. A longer term goal is to characterize the genes which are induced or activated in presence of heavy metals. These genes ideally would be specific to metals and would provide a measurable and linear response to toxic metal exposure. The ultimate goal of this project is to

develop bioassays for bioavailable toxic metal ions at environmentally relevant concentrations.

*C. reinhardtii*, a widely used experimental system, is the organism of choice for the current study. This alga is employed to test for pollutants in water and soils and is expected to be tolerant to potential toxic components (e.g. fungal, antimicrobial and phytotoxins) that could affect the response of the organism to toxic metal ions. Tools and standard techniques have been developed over the years for genetic analysis, DNA isolation and reintroduction of genes into the genome (Rochaix *et al.*, 1999) and the full genomic DNA sequence has been available since February 2003. This photosynthetic microorganism is easier to maintain, culture and manipulate than plants. Study of the defense system of *C. reinhardtii* against toxic metals could lead to a better understanding of the defense system of land plants.

In this project, a transgenic alga with a reporter gene under the control of regulatory elements that respond to toxic metal exposure was generated. The strain was tested for the specificity of its response to metals. It was also analyzed by Northern blotting, RNase protection assays and real time PCR to determine the linearity of the reporter gene's response to increasing metal concentrations.

## CHAPTER 2. MATERIALS AND METHODS

### 2.1 Preparation of the transformants

#### 2.1.1 *Arg7* cDNA expression cassette used for transformation

The plasmid pRb-ARGs (Figure 1A) (Auchincloss *et al.*, 1999) was digested with *SphI* to remove the distal 671 bp of the *RbcS2* promoter (785 bp). The band at 5.7 Kb was gel-purified using the Phenol-Freeze-Fracture Method (Bewsey, 1991) (Figure 1B). After purification the product was quantified using the Syngene GeneTools software and then used for transformation.

#### 2.1.2 Transformation and screening

Cultures of *C. reinhardtii* CC1618 (Table 2) were grown in Tris-Acetate-Phosphate (TAP) medium (186 mM NH<sub>4</sub>Cl, 8,5 uM of CaCl<sub>2</sub>·4H<sub>2</sub>O, 10 uM of MgSO<sub>4</sub>·7H<sub>2</sub>O, 14,8 uM, KH<sub>2</sub>PO<sub>4</sub>, 25 mM K<sub>2</sub>HPO<sub>4</sub>, 10 uM Tris and 0.5 mM CH<sub>3</sub>COOH) plus trace metals solution (11.4 ug H<sub>3</sub>BO<sub>3</sub>, 22 ug ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.6 ug Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 1.1 ug Na Molybdate·2H<sub>2</sub>O, 1.6 ug CuSO<sub>4</sub>·5H<sub>2</sub>O, 5.06 ug MnCl<sub>2</sub>·4H<sub>2</sub>O, 4.9 ug FeSO<sub>4</sub>·7H<sub>2</sub>O and 50 ug Na<sub>2</sub>EDTA dissolved in 1 ml of water) (Gorman, 1965). The concentration of trace metals in molarity is indeterminate because of discarded precipitate. The TAP solution (also called “old” TAP to distinguish it from another TAP medium) was supplemented with 55 ug/ul of L-arginine. The cultures were grown to a density of 2x10<sup>6</sup> cells/ml then harvested by centrifugation at 3800 x g for 5 min. The pellet was resuspended gently in 1/100 volume of TAP supplied with arginine and agitated at

room temperature for 2 hours. To a test tube containing 300 mg of sterile glass beads (Thomas) (pretreated by washing with acid and baked for a few hours at 400° F), 300 ul of cell solution, 100 ul of 20 % (w/v) PEG and 1 ug of gel purified plasmid were added. The tube was vortexed for 30 sec at top speed, left at room temperature for 6 hours and then the mixture was plated on agar plates with TAP medium containing nickel or cadmium (Kindle, 1990). The transformants appeared after 2-3 weeks. The agar-solidified medium in petri plates contained sublethal concentrations of nickel or cadmium. To determine which concentration of metal is sublethal, three strains (CC424, CC425 and CC1618) were grown on plates with different nickel and cadmium concentrations (Table 1). A visual determination based on the growth quantity and the chlorosis effect was used as a guide for cell viability. The transformation frequency dropped when the concentration of nickel was higher than 100 uM and the concentration of cadmium was higher than 150 uM. Concentrations of 100 uM of nickel and 150 uM of cadmium were found to be sublethal and these concentrations were used for the selection. The cells lines were patched in a 10 x 10 grid using sterile toothpicks in TAP media solidified with agar (1.5%). These were allowed to grow for XX days at 24oC and then replica-plated to TAP medium with or without the metal but lacking arginine. 11000 transformants that grew on plates with metal but not on plates without metal were selected for further analysis.

### **2.1.2 Attempted trials to generate transgenic *C. reinhardtii* strains**

In the first approach, *C. reinhardtii* CC1618 was transformed as described previously with the *Ars* gene as a reporter gene and the *Arg7* cDNA plasmid as selectable marker (Hostos *et al.*, 1988). The colonies were sprayed with the chromogenic substrate, 5-bromo-4-chloro-indolylsulphate until development of blue color.

In the second attempt, cultures of *C. reinhardtii* *pf-14 arg7*, was transformed as described previously with promoterless *Rsp3* as a reporter gene and with a linearized *Arg 7.8* vector containing the *Arg7* genomic gene as a selection marker (Haring and Beck, 1997). Cells were tested for expression of the reporter gene by swimming function in presence of metal.

### **2.2 RNA extraction from *C. reinhardtii* and poly-(A)<sup>+</sup> isolation**

Two methods for extraction of RNA were used: for small cultures (15 ml), the TRIZOL method was used and for large cultures (250 ml) the polytron (to grind cells) was used. For both cultures volumes, *C. reinhardtii* were grown to high density ( $1 \times 10^7$  cells/ml) in TAP media with arginine and harvested by centrifugation at 3800 x g for 5 min. The pellet was resuspended in TE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 8) and centrifuged again as previously. The pellet was frozen at -80 °C until RNA was ready to be extracted.

For 250 ml cultures, 5 ml of RNA extraction buffer (50 mM Hepes-NaOH, 100 mM NaCl, pH 7.5) and 5 ml of phenol saturated in extraction buffer were added to the frozen cell pellet and homogenized with the Polytron at speed 6

for 45 sec. After addition of 1 ml 1% (w/v) SDS the sample was vortexed for 20 sec and centrifuged at 2000 x g for 10 min at room temperature. The aqueous phase was recovered and extracted twice with 5 ml of (25:24:1, v/v) phenol/chloroform/isoamyl alcohol saturated in extraction buffer, and then twice with 5 ml of (24:1, v/v) chloroform/isoamyl alcohol. The aqueous phase was recovered and the RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol and incubating for 30 min at -20°C. Samples were centrifuged at 15000 x g at 4°C for 20 min and the pellets were dissolved in water.

For the 15 ml cultures, 0.5 ml of TRIZOL reagent (Sigma) was added to the frozen cell pellet and vortexed twice for 15 sec. Then 100 mg of sterile glass beads (Thomas), which had been washed with acid and baked for few hours at 400° F, were added and vortexed for 15 sec at high speed. After incubation (5 min at room temperature) the samples were centrifuged for 15 min at 15000 x g at 4°C followed by another incubation of 5 min at room temperature. After addition of 100 ul of chloroform, the mixture was vortexed for another 15 sec, incubated for 5 min at room temperature and centrifuged at 15000 x g for 15 min at 4°C. The aqueous phase was recovered and extracted once with 500 ul of (25:24:1, v/v) phenol/chloroform/isoamyl alcohol and extracted twice with 500 ul of (24:1, v/v) chloroform/isoamyl alcohol. The supernatant was collected and the RNA was precipitated by adding 250 ul of 2-propanol. After 5 min incubation the samples were centrifuged at 15000 x g for 10 min 4°C. The aqueous phase was recovered and the RNA was precipitated by adding 500 ul of 95% ethanol and incubating for



30 min at -20°C. The samples were centrifuged at 5800 x g for 5 min at 4°C. The pellet was dissolved in water and RNA concentration was quantified by measuring the OD<sub>260</sub> with a spectrophotometer.

To isolate poly-(A)<sup>+</sup> RNA, a solution containing 1.5 mg of total RNA was incubated at 65°C for 5 min and then chilled on ice. Once removed from the ice an equal volume of 2 X column-loading buffer (40 mM Tris-Cl, (pH 7.6), 1 mM NaCl, 2 mM EDTA (pH 8.0), 0.2 % (w/v) sodium lauryl sarcosinate) was added to each RNA sample. Each sample was applied to a column containing 200 mg of oligo(dT)-cellulose pre-equilibrated with 1 X column-loading buffer. Once the solution passed through, the column was washed with 1 volume of column loading buffer. The flow-through fraction was collected and incubated at 65°C for 5 min then applied a second time to the column followed by a washing step with 10 ml of column loading-buffer. Poly-(A)<sup>+</sup> RNA was eluted from the column with 600 ul of elution buffer (10 mM Tris-Cl (pH 7.6), 1 mM EDTA (pH8.0), 0.05% (w/v) SDS). The poly-(A)<sup>+</sup> RNA was precipitated by addition of 600 ul of 95% ethanol, and incubated for 30 min at -20°C. The samples were centrifuged at 5800 x g rpm for 5 min at 4°C. The pellet was dissolved in RNase-free water and quantified by measuring the OD<sub>260</sub> with a spectrophotometer. Poly-(A)<sup>+</sup> RNA was also purified from total RNA with PolyAtract RNA isolation System III (Promega) following the instructions of the manufacturer. The purified poly-(A)<sup>+</sup> RNA was analyzed for purity and quantity with the Agilent 2100 Bioanalyzer.

### 2.3 Southern analysis

Southern blot analysis was carried out with 10 ug of total DNA according to Sambrook *et al.* (1989) with some modifications. The DNA samples (20 ug) were digested overnight with *Apal* and *Sacl* enzymes, treated with 1 unit Rnase A for 30 minutes at 37°C, extracted with one volume of (25:24:1, v/v) phenol/chloroform/isoamyl alcohol and twice with one volume of chloroform. Digested DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol. After centrifugation the pellet was dissolved in 30 ul of water and quantified by measuring the OD<sub>260</sub> with a spectrophotometer. 10 ug of digested and purified DNA was separated by electrophoresis on a 0.8% agarose gel containing 1X TBE (90 mM Tris, 90 mM H<sub>3</sub>BO<sub>3</sub> and 2 mM EDTA, pH 8) at 3,5 V/cm until the bromophenol blue loading dye migrated approximately ¼ of the gel length. The agarose gel was rinsed briefly with water, soaked in 5X volume of denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 45 min and rinsed briefly with water to remove the denaturation solution. The gel was then soaked twice for 15 min in neutralization solution (1 M Tris (pH 7.4), 1.5 M NaCl) and rinsed briefly with water before transfer. The DNA was transferred to a Hybond-N nylon filter (Amersham) by capillary blotting with 20 X SSC (3 M NaCl and 0.3 M sodium citrate) overnight and the DNA was cross-linked using 1.2 J in the Stratagene Stratalinker as per the manufacturer's specifications. After transfer and cross-linking the membrane was washed with 6 X SSC (0.9 M NaCl and 0.09M sodium citrate). The membranes were prehybridized at 42°C for 2 hours in hybridization solution containing 6 X SSC, 0.5% (w/v) SDS, 50%

formamide, 5 X Denhardt's solution (0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) Ficoll) and 25 mg sonicated denatured salmon sperm DNA. The Southern blot was hybridized with an *ARG7* probe prepared from the plasmid that had been digested with *Apa1* and *Sac1*, as described in section 2.1.1. To radiolabel the probe, 100 ng of DNA and 100 ng of random hexamer primer were heated for 2 min and chilled on ice. 1X EcoPol buffer (Amersham), 75 uCi of [ $\alpha^{32}\text{P}$ ] dATP (3000 Ci/mmol), 0.5 mM dNTPs, Klenow fragment enzyme and water up to 25 ul were added and the mixture was left at room temperature for 1 hour. Unincorporated dNTPs were removed from the radiolabeled probe by passing the reaction mixture through a Sephadex G50 column. The labeled probe was heat denatured at 95°C for 2 min and added to the hybridization solution. The membranes were hybridized at 65°C for 16 hours in the same solution used for prehybridization and then washed twice with 2 X SSC and 0.5% (w/v) SDS at room temperature for 15 min and once with 2 X SSC, 0.1% (w/v) SDS at room temperature for 15 min. The membranes were then incubated for 4 hours at 65°C with 0.1% SSC, 0.1 % (w/v) SDS. Hybridization signals were revealed by autoradiography.

#### **2.4 Northern analysis**

Northern blot analysis was carried out with 20 ug of total RNA or 5 ug of poly-(A)<sup>+</sup> RNA according to Sambrook *et al.* (1989) with some modifications. The RNA samples were precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol. After centrifugation at 15000 x g for 15 min the

pellet was dissolved in 200  $\mu$ l of 10 X gel loading dye (500 mM Hepes (pH 7.5), 10 mM EDTA (pH 8.0), 900  $\mu$ l formamide, 40  $\mu$ l of bromophenol blue (20 mg/ml) and 300  $\mu$ l formaldehyde). The samples were heated to 65°C for 10 min and then chilled on ice. The RNA was separated by electrophoresis on a 0.8% agarose gel containing 50 mM Hepes (pH 7.5), 1 mM EDTA (pH 8.0) and 18 M formaldehyde with a running buffer consisting of 50 mM Hepes (pH 7.5) and 1 mM EDTA (pH 8.0). Electrophoresis was carried with 4.6 v/cm until the dye migrated approximately  $\frac{3}{4}$  of the length of the gel. The agarose gel was then soaked in 10 X SSC (3 M NaCl, 0.3 M sodium citrate) for 10 min and the RNA was transferred to a Hybond-N nylon filter (Amersham) by capillary blotting with 20 X SSC overnight. After crosslinking in the Stratagene Stratalinker the membrane was soaked in methylene blue solution in order to visualize the successful transfer of RNA. Northern blots were hybridized with an *ARG7* probe that was prepared by random priming 100 ng of digested plasmid as described above (Southern analysis, section 2.3). After blotting the membrane was washed with 6 X SSC. The Northern blots were prehybridized at 42°C for 2 hours in hybridization solution containing 6 X SSC (0.9 M NaCl and 0.09M sodium citrate), 0.5% (w/v) SDS, 50% formamide, 5 X Denhardt's solution ( 0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) Ficoll) and 25 mg sonicated denatured salmon sperm DNA. The labeled probe was heat denatured at 95°C for 2 min and added to the hybridization solution. The Northern blots were hybridized at 65°C for 16 hours and then washed twice with 2 X SSC and 0.1% (w/v) SDS at room temperature for 15 min and 4 times with 0.2

X SSC and 0.1% (w/v) SDS at 65°C. Hybridization signals were revealed by autoradiography.

## 2.5 RNase protection assay

The RNase protection assay was performed according to established protocols (Sambrook, 1989) with a few modifications. The probe was prepared by cloning a 353 bp fragment cut with *Sall* and *SacI* from the vector pARG7.8 phi3 (Debuchy *et al.*, 1989) containing the genomic *Arg7* gene into the pBS vector (Stratagene). The new vector (pBS+RNAprobe) was cut with the restriction enzyme *EcoRI*. Run-off transcription by T3 RNA polymerase generated a RNA probe of 392 bp (Figure 2). To radiolabel the probe 0.5 ug of *EcoRI* digested and phenol/chloroform purified pBS+RNAprobe vector plus 0.2 M DTT, 5 mM ribonucleotide solution (rA, rC, rU), 100 uM of 800 uCi [ $\alpha$ -<sup>32</sup>P]GTP, 1 X transcription buffer (0.04 M Tris-Cl (pH7.5), 0.01 M NaCl, 6 mM MgCl<sub>2</sub>, 2 mM spermidine), 20 units of RNaseOUT and 24 units of T3 RNA polymerase were incubated for 1 hour at 37°C followed by addition of 1 unit of RNase-free DNase and incubation for another 10 min at 37°C. After addition of 20 ug of carrier tRNA the samples were extracted once with (25:24:1, v/v) phenol/chloroform/isoamyl alcohol, then extracted twice with (24:1, v/v) chloroform/isoamyl alcohol and purified by passing through a spin column containing Sephadex G50 to remove the unincorporated nucleotides. The RNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate and 95%

ethanol. The RNA samples were combined with  $2 \times 10^5$  cpm of probe, precipitated with 3 M sodium acetate and 2.5 volumes of 95% ethanol, and the pellet was dissolved in 30  $\mu$ l hybridization buffer (40 mM Pipes (pH 6.8), 1 mM EDTA (pH 8.0), 0.4 M NaCl, 80% deionized formamide). The samples were incubated at 85°C for 10 min then incubated for 8 hours at 55°C. The hybridization mixture was chilled and after addition of 300  $\mu$ l of RNase digestion mix (300 mM NaCl, 10 mM Tris-Cl (pH 7.4), 5 mM EDTA (pH 7.5), 40  $\mu$ g/ml RNase A and 4 units of RNase T1) the samples were incubated for 1 hour at 30°C. After the digestion step the samples were incubated for another 30 min at 37°C in the presence of 14  $\mu$ l proteinase K digestion mixture (20% (w/v) SDS, 100 mg proteinase K). The samples were extracted once with (25:24, v/v) phenol/chloroform, twice with chloroform, and precipitated by addition of 20  $\mu$ g carrier tRNA and 2.5 volumes of 95% ethanol. The pellet was dissolved in RNA loading dye and electrophoresed on a 6% denaturing polyacrylamide gel.

## **2.6 New media developed for induction**

This medium was developed to expose the cultures to toxic metal ions then to extract RNA for Northern analysis. The new medium contains: 186  $\mu$ M of  $\text{NH}_4\text{NO}_3$ , 8,5  $\mu$ M of  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 10  $\mu$ M of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 14,8  $\mu$ M  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{K}_2\text{HPO}_4$ , 10  $\mu$ M Tris and 50 mM  $\text{HNO}_3$ . The traces metals solution is composed of 16.3  $\mu$ M  $\text{H}_3\text{BO}_3$ , 3.5  $\mu$ M  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.8  $\mu$ M  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 0.2  $\mu$ M Na Molybdate  $\cdot 2\text{H}_2\text{O}$ , 0.3  $\mu$ M  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 2  $\mu$ M  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 3.6  $\mu$ M  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 17.1  $\mu$ M sodium citrate  $\cdot 2\text{H}_2\text{O}$ . This medium contains nitrate

salts instead of chloride salts (for example  $\text{NH}_4\text{NO}_3$  instead of  $\text{NH}_4\text{Cl}$ ) because the bioavailability of metal is increased or decreased in the presence of  $\text{Cl}^-$  ions. This medium also contains sodium citrate (chelating value  $\log K=4.98$ ) instead of EDTA (chelating value is  $\log K=18.2$ ), the latter being a 3.6-fold stronger chelator than citrate and able to complex most of the metals present in new TAP medium solution. In this medium 90% of the subsequently added  $\text{Cd}(\text{NO}_3)_2$  is present as  $\text{Cd}^{2+}$ .

## **2.7 Real time RT-PCR**

### **2.7.1 Preparation of the cDNA from RNA**

Cultures of *C. reinhardtii* (Ni70, CC1618, and CC1618+P) were grown in “old” TAP medium supplemented with 55  $\mu\text{g}/\mu\text{l}$  of L-arginine until a density of  $3 \times 10^6$  cells/ml was attained. Cells were then harvested by centrifugation at 2500 x g for 10 min. For induction with metal, growth medium were prepared without trace metals and chelating compound and this media was called TAP-trace metals. The cells were washed twice with TAP-trace metals solution and resuspended in 200 ml of TAP-trace metals solution. The cultures were induced with different concentrations of metal for 2 hours then centrifuged at 2500 x g for 10 min. The pellet was resuspended in 4 ml of TE buffer prepared with DEPC-treated water and centrifuged for 2 min at 5000 x g. The pellet was frozen at  $-80^\circ\text{C}$  until RNA extraction was ready to be performed. The procedure for RNA extraction using the TRIZOL method was described previously (Section 2.2). The RNA samples were DNase treated with Optimal Column DNase (Qiagen) and purified with the

Qiagen RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA was quantified by measuring the OD<sub>260</sub> with a spectrophotometer and quality was determined using the Agilent 2100 Bioanalyzer. The reverse transcription reaction of the RNA into cDNA was performed by random priming using 0.5 ug of total RNA, 250 ng hexanucleotides, 10 umol dNTPs and water up to 12.5 ul, placed 5 min at 70°C and 5 min at 4°C. Then 1X transcription buffer (Stratagene), 20 umol DTT, 23 units RNaseOUT, 200 units Reverse Transcriptase and water up to 20 ul were added and the mixture was placed 10 min at 25°C, 50 min at 42°C and 15 min at 70°C. Negative controls were prepared by omitting the reverse transcriptase.

### **2.7.2 Quantitative analysis of real time RT-PCR data**

Quantitative fluorogenic RT-PCR was performed in an Applied Biosystems 7900 HT Sequence Detection System. The oligos used for the PCR reactions (to amplify the *Arg7* gene and the control genes *Pdk*, *Tub* and *PsaB*) were designed using the primer/probe design program, Primer Express (Applied Biosystems) and are listed in Table 3. For every new pair of oligos a standard curve was performed to analyze the efficiency of the amplification. The PCR reactions for the standard curve were performed with 4-fold, 16-fold, 64-fold and 256-fold serial dilution of the original reverse transcription (RT) preparation. All reactions were performed using the SYBR Green Universal PCR Master Mix (including AmpliTaq Gold DNA Polymerase, SYBR Green dye and ROX reference dye) with a 16-fold dilution of the reverse transcription reaction mixture



and 3  $\mu\text{M}$  of primers in a 20  $\mu\text{l}$  final volume. Cycling conditions were 2 min at 50°C, 10 min at 95°C followed by 50 two-temperature cycles (15 sec at 95°C and 1 min at 60°C). Data were analyzed using the sequence detection software supplied with the instrument (SDS 2.0).

## CHAPTER 3. RESULTS

### 3.1 Generation of transgenic *C. reinhardtii* strains that express a reporter gene in response to metal exposure

Foreign genes are efficiently silenced in the *C. reinhardtii* nucleus (Cerutti *et al.*, 1997). Therefore, our choices of reporter genes for promoter trapping screens were restricted to the use of *C. reinhardtii* genes whose expression can be monitored with a simple assay. We initially attempted to use two established reporter genes for promoter trapping screens but difficulties arose that precluded their usefulness.

The first system involved the introduction of a promoterless arylsulphatase gene (*Ars*). Arylsulphatase is secreted to scavenge sulfate in response to sulfur deprivation in *C. reinhardtii* (Hostos *et al.*, 1988). Its activity can be detected by the chromogenic substrate, 5-bromo-4-chloro-indolylsulphate, which when cleaved releases the sulphate and a blue product. An *arg7* mutant strain was cotransformed with a promoterless reporter gene (*Ars*) and a selectable transformation marker, the *Arg7* cDNA expression cassette (Auchincloss *et al.*, 1999), which confers arginine prototrophy. Several hundred colonies of the *arg*<sup>+</sup> transformants were sprayed with a solution of 5-bromo-4-chloro-indolylsulphate and observed for the development of a blue color that reveals presence of arylsulphatase activity. Of these c.a. 3-5% were expected to have insertions that result in expression of the *Ars* gene under the control of transcriptional regulatory sequences adjacent to the insertion site and, thus, generate a blue colony color in the presence of the chromogenic substrate. The initial trials were not conclusive

because the blue color developed only after 16 hours and for some strains the result was ambiguous. Moreover, as high expression of the *Ars* gene is needed to see a signal, only transformants that have the *Ars* gene inserted optimally for transcription close to a random promoter can generate a detectable signal.

A second promoter trap system has been developed to identify genes that are induced by nitrogen starvation (Haring and Beck, 1997). It involves the introduction of the gene encoding the radial spoke protein (*Rsp3*), which is required for functional flagella and motility. Expression of a transformed copy of *Rsp3* restores motility to a mutant strain for this locus, which has paralyzed flagella. The advantage of this system for identification of metal-regulated genes was that the selection could be performed in liquid media where metal speciation can be more precisely controlled than in media solidified with agar. Agar can contribute to the chelation of metal ions. The progenitor strain, (*pf-14 arg7*, Haring and Beck, 1997), was transformed with a linearized *Arg 7.8* vector containing the *Arg7* genomic gene as a selection marker for transformation and the promoterless *Rsp3* as a reporter gene (Haring and Beck, 1997). *rps3* mutant cells sediment to the bottom of the culture while transformants that express *Rsp3*, due to presence of a promoter near the insertion site, are motile and found throughout the culture. We had hoped that a major advantage of this system would be the ability to select *en masse* for rare transformants that swim only in the presence of a metal. Thousands of transformants would be allowed to sink or swim in the same culture lacking a toxic trace metal. The supernatant fraction with "swimmers" (*Rsp3* expressers) would be removed from the "sinkers" (non-

expressers) in the pellet. These *Rsp3* non-expressing “sinkers” were then to be resuspended in a medium containing a metal of interest and the “sink or swim” assay repeated. Cells that swim (express *Rsp3*) only in the presence of metal were to be selected by their ability to swim and enter the supernatant fraction. Unfortunately, a high background of mutant cells was detected in the upper “swimming” fraction. These *rsp3* mutant “floaters” precluded our ability to select for rare “swimmers”. Indeed, the original report of this promoter trapping method involved the testing of individual transformant lines, and thus was extremely labor-intensive. Moreover, using both a selectable marker (*Ars*) and a reporter gene (*Rsp3*), resulted in a high background of reporter gene insertions that were not expressed.

### **3.2 Development of transgenic *C. reinhardtii* strains that report bioavailable cadmium and nickel**

An *Arg7* minigene cassette (Figure 1B) that circumvents the aforementioned problems was used in a promoter-trapping screen. In this system, the same gene is used as reporter and transformation marker and, thus, only transformants that express the reporter gene are obtained.

*Arg7* encodes argininosuccinate lyase, the last enzyme in the *de novo* arginine biosynthetic pathway. This *Arg7* cDNA cassette complements *arg7* mutations suggesting that it could be used in experiments designed to identify promoters under certain growth conditions. A plasmid (pRb-ARGs), with the *RbcS2* promoter driving expression of the *Arg7* cDNA cassette with its 5'UTR, coding sequence, and the genomic 3' end to ensure proper 3' end formation and

poly-adenylation of the transcript, was used for this promoter trap experiment (Figure 1A, Auchincloss *et al.*, 1999). By using the same gene (*Arg7*) as a selectable marker and a reporter gene, the problem of obtaining a high background of false positives was resolved. As arginine prototrophy was used as to indicate expression of the reporter gene, transformants had to be selected in the presence of sublethal concentrations of the metal in order to induce the reporter gene. Transformants that express the *Arg7* cDNA cassette were selected and subsequently screened on media lacking metal and in media with metal. Transformants that were *arg*<sup>+</sup> on media with metal but not on media lacking metal were selected for further analysis.

A strain carrying the *arg7-8* mutation (CC1618) was transformed with a *SphI* digested pRb-ARGs plasmid (Figure 1A). Linearizing the plasmid with *SphI* removed the distal 671 bp of the *RbcS2* promoter, leaving 119 bp of sequence upstream of the 5' end *Arg7* cDNA (Figure 1B). The remaining 119 bp contained the *RbcS2* transcription start site and the start codon but not the CAATT box. The base pairs upstream of the 5' end of *Arg7* that were retained acted as a buffer region against removal of *Arg7* 5' leader or coding sequences by exonuclease digestion before integration of this DNA fragment into the genome. The 26 bp upstream of the *RbcS2* transcription initiation site is insufficient to drive expression because a previous study showed inefficient expression of a reporter gene from the proximal 180 bp of the *RbcS2* promoter (Cerutti *et al.*, 1997).

Analysis of the integrated transforming DNA into the genome suggests that the insertion of linear plasmids occurs preferentially through non-homologous recombination via the ends. Cerutti and coworkers constructed a cassette containing the *RbcS2* promoter linked to the *aadA* gene (a bacterial gene that confers streptomycin and spectinomycin resistance) to study expression of foreign genes in *C. reinhardtii*. Transformation efficiency dropped to 40% when the cassette contained only 180 bp of the *RbcS2* promoter versus full promoter. Southern blotting experiments demonstrated that the plasmid was inserted into the genome with terminal deletions of about 100bp (Cerutti *et al.*, 1997).

To select the strains that grow only in the presence of metal, it was necessary to determine a metal concentration that induces a response but is sublethal. Three strains (CC424, CC425 and CC1618, Table 1) were grown on media (solidified with agar in petri plates) with different nickel and cadmium concentrations (Materials and Methods, section 2.1.2). Concentrations of 100  $\mu$ M of nickel and 150  $\mu$ M of cadmium were found to be sublethal and used for selection of putative transformants (see Materials and Methods). These metal concentrations should induce the tolerance mechanism (i.e. metal regulated genes). The transformation efficiencies were approximately 10-fold lower than in the absence of the metal and the colonies had a paler green color than colonies of transformants selected in the absence of additional metal (data not shown).

*Arg*<sup>+</sup> transformed strains were obtained on medium containing the sublethal concentrations of either nickel or cadmium. The transformants were maintained on TAP medium supplied with arginine prior to screening. These candidates were

retested for arginine prototrophy (*Arg7* cDNA expression) by replica plating to TAP medium with or without metal (150  $\mu$ M Cd or 100  $\mu$ M Ni). Strains that were  $\text{arg}^+$  in presence of metal but not in the absence of metal were picked for further analysis. Four such metal-responsive strains were obtained from the 4,500 transformant strains selected for expression of the *Arg7* cDNA cassette in the presence of cadmium. They were named Cd98, Cd150, Cd155, and Cd3600 (Table 2). Similarly, four strains were obtained from the 6,500 transformant strains selected for expression of the *Arg7* cDNA cassette in the presence of nickel. They were named Ni70, Ni131, Ni713 and Ni203 (Table 2, Figure 3). From visual inspection of colony sizes, Ni70 and Cd3600 grow faster in the presence of metal on TAP medium without arginine, than the other strains and failed to grow on TAP medium with neither metal nor arginine. These strains are good candidates for having an insertion of the reporter gene in a region of the genome that is near a metal-regulated promoter because they are  $\text{arg}^-$  in the absence of metal. Thus, Ni70 and Cd3600 were extensively studied in subsequent experiments. As a positive control CC1618 was also transformed with the uncut pRb-ARGs to obtain an arginine prototroph transformant in which the *RbcS2* promoter drives the expression of the *Arg7* cDNA cassette. This strain was called CC1618+P.

The specificity of the responses of Ni70 and Cd3600 to metals was tested again by testing for arginine prototrophy in the presence of 150  $\mu$ M zinc, copper, cobalt, silver, beryllium, lead, calcium or 70  $\mu$ M iron. Both strains are  $\text{arg}^+$  in the presence of all of these metals. Figure 4 shows these results for iron and silver.

The induction of the  $arg^+$  phenotype of Ni70 and Cd3600 by calcium was surprising (data not shown). Calcium might induce expression of the *Arg7* cDNA by displacing the equilibrium of metal ion bioavailability; a higher quantity of other free metal ions is available due to the chelation of the calcium.

### **3.3 Metal regulated transformants have a single insertion of *Arg7* cDNA**

In order to determine the number of insertions of the *Arg7* cDNA cassette present in each transformant, Southern analysis on genomic DNA was performed. Genomic DNA was prepared from each strain and digested with *Apal* and *Sacl*. These enzymes do not cut within the transformed DNA and thus yield a single fragment that hybridizes to the *ARG7* probe (Materials and Methods, section 2.3). A restriction fragment derived from the endogenous (and mutant) *Arg7* locus was detected in all strains, including the non-transformed progenitor strain (Figure 5). In the digests of the genomic DNA of Ni70 and Cd3600 a single additional band was detected indicating that they harbor a single insertion of the reporter gene. The additional multiple bands detected in genomic restriction digests of DNA from the other transformants (e.g. Ni713) suggest that more than one copy of the *Arg7* cDNA cassette have been integrated. Integration of transformed DNA into the *C. reinhardtii* nuclear genome occurs into random sites by non-homologous recombination. Integration by homologous recombination does occur, but a frequency less than 0.7% (Sodeinde and Kindle, 1993). Thus, insertions at the *Arg7* locus were not expected and, indeed, none were detected.



### **3.4 A modified medium was developed to minimize the formation of non-bioavailable species of Ni and Cd**

It was necessary to expose cells to metal under conditions in which the metal is bioavailable. As many components of TAP medium can chelate metals (e.g. Cl<sup>-</sup>, EDTA) metals and thereby remove them from the bioavailable metal pool, a modified culture medium that prevents the formation of non-bioavailable species of Cd and Ni was developed (see Materials and Methods, section 2.6). For the following experiments the modified TAP medium was used in which EDTA was replaced by citrate and NH<sub>4</sub>Cl was replaced with NH<sub>4</sub>NO<sub>3</sub>.

### **3.5 A novel *Arg7* transcript is induced by metal in Ni70 and Cd3600**

Northern analysis was performed to determine whether the expression of the reporter gene in Ni70 and Cd3600 is induced by metal exposure. RNA was isolated from cultures incubated in the presence of either Cd (Cd3600) or Ni (Ni70) in the modified TAP medium. Samples of total RNA were electrophoresed on agarose gel, transferred to membrane, and hybridized to the *ARG7* probe (Materials and Methods, section 2.4). In all strains tested, including the non-transformed progenitor strain CC1681 (data not shown), the endogenous *Arg7* mRNA was detected (Figure 6). Ni70 and Cd3600 express an additional mRNA species only in the presence of metal, which was attributed to the introduced *Arg7* cDNA cassette. *Arg7* is a single copy gene in this organism and the size of its mRNA transcript is 2.3 kb (Auchincloss *et al.*, 1999). The bands corresponding to inserted reporter gene are of another size probably because the transcript is fused with the transcription unit at the insertion site. The smaller size

of the novel *Arg7* transcript in Cd3600 is more difficult to explain. The minimal coding sequence required for argininosuccinate lyase activity has not been determined. In Cd3600 a partially deleted copy of the *mingene* might still encode a functional enzyme. It is even possible that the insertion results in a fusion protein, due to an in-frame insertion of the cDNA into the coding region of a metal-regulated gene. To normalize for the amount of RNA loaded in each lane, rRNA on the filter was stained with methylene blue. The band intensity of the endogenous *Arg7* gene was a second indicator to verify the quantity of RNA loaded in each lane. Thus, both Ni70 and Cd3600 appear to induce the expression of the reporter gene in response to metal exposure.

In an effort to determine whether these responses are specific to metal exposure, or if other stresses can induce them, Northern analysis was performed on strains Ni70 and Cd3600 that had been exposed to high salt, paraquat, heat, and lead. These strains were incubated with 300 mM NaCl (Meijer *et al.*, 2001) or 1  $\mu$ M paraquat for 2 hours (Irihimovitch and Shapira, 2000). Heat shock was induced by rapidly shifting cultures from physiological temperature (24°C) to a waterbath at 41°C for 1 hour (Schroda *et al.*, 1999). However, the results were ambiguous (data not shown).

RNase protection can be a more sensitive assay for detecting and quantifying transcript abundance than Northern blot analysis. We attempted to use this technique to quantify the level of the transcript from the *Arg7* cDNA in the presence and absence of metal. The experiment was performed with total and poly-(A)<sup>+</sup> RNA from cells that were treated or not treated with cadmium. The

RNA probe was derived from the 5' terminus of the *Arg7* gene, in the pArg7.8 plasmid (Debuchy *et al.*, 1989) which contains the genomic *Arg7* gene (Figure 2). Hybridization of the probe to the endogenous *Arg7* protects 262 nt of transcript, while hybridization of the probe to the inserted reporter gene protects 139 nt of transcript. The level of the endogenous *Arg7* mRNA serves as a control for the quantity of RNA loaded. Strain Ni70 exposed to metal produced bands corresponding to endogenous *Arg7* protected fragment (133 nt instead of 139 nt) and the inserted reporter gene (262 nt) at the expected position. Control strain, CC1618P, also produced two bands corresponding to endogenous and inserted reporter gene. However, neither the Ni70 strains nor the control strain (CC1618) had the expected band for endogenous *Arg7* in the absence of metal (Figure 7). Other unexpected protection products were observed which range between 150 nt and 250 nt.

### **3.6 Real time reverse transcription PCR (RT-PCR)**

Although encouraging results were obtained from Northern analyses (Figure 6), the signal from the *Arg7* mRNAs was extremely weak, requiring maximum sensitivity of the technique. In addition, the RNase protection assay was not sufficiently sensitive to detect the *Arg7* cDNA transcript in the promoter-trapped transformants (Figure 7). This sensitivity problem was resolved by developing a real time RT-PCR assay for the transcript of the reporter gene. Only strain Ni70 was analyzed, but with this assay now established similar analysis should be performed on strain Cd3600.

Another problem that was encountered in several trials of the Northern analysis was a lack of induction in the accumulation of the *Arg7* mRNA in response to exposure to Ni or Cd. Similarly, in early trials of the RT-PCR assay expression of the reporter gene was not induced (data not shown). As EDTA in the TAP medium could chelate much of the Ni or Cd added to induce expression, cultures were grown TAP medium and then transferred to TAP without trace element solution, which contains no EDTA. This new medium for induction contains no trace metals and no EDTA. This resulted in reproducible induction of the *Arg7* cDNA expression in the three trials performed using the RT-PCR method on two independently prepared sets of RNA.

Using the real time RT-PCR assay (Freeman et al., 1999), the level of the reporter gene transcript was quantified relative to a known control mRNA. A relative quantification consisted of comparing a sample (Ni 70 exposed to metal) to the calibrator (Ni70 non-exposed). During the RT-PCR assay the target Ct is compared directly with the calibrator Ct and is recorded as containing more or less mRNA. A passive internal reference (ex. ROX) is used to normalize non-PCR related fluorescence fluctuations. Normalizing with a passive internal reference minimizes well-to-well variability that can result from a variety of causes, such as pipetting errors and sample evaporation. Other, specific errors are due to variation in the amount of starting material between samples. To minimize these errors, and correct for sample to sample variations, a cellular RNA that serves as an internal standard is amplified simultaneously. This internal standard ideally should be expressed at constant level, at roughly the same level as the

transcript of the inserted *Arg7* reporter minigene. Three genes were used as internal standards for this analysis; *Pdk* which encodes pyruvate dehydrogenase kinase (Ferris *et al.*, 2002), *Tub* which encodes epsilon-tubulin protein (Dutcher *et al.*, 2002) and *PsaB* which encodes P700 chlorophyll A (Nozaki *et al.*, 2000).

First strand cDNA synthesis was primed with oligos (Table 3) and extended with reverse transcriptase. These cDNAs were then amplified with oligonucleotide primers that hybridize to the mRNA of interest. PCR products were detected with the fluorescent dye SYBR Green. The unbound dye exhibits little fluorescence, and fluorescence occurs when the dye binds to double-stranded DNA of the PCR products. This increase in fluorescence is monitored in real time during amplification and reflects the amount of PCR product. The Cd concentrations tested were  $10^{-8}$  M,  $10^{-6}$  M,  $10^{-5}$  M and  $10^{-4}$  M. Cultures were also treated with Ni at  $3 \times 10^{-4}$  M and  $3 \times 10^{-6}$  M. RT-PCR was performed with one pair of oligos (*Arg1*) for the reporter gene and three other pairs of oligos for control genes *Tub*, *Pdk* and *PsaB* (Table 3). To avoid detection of PCR products from genomic DNA, the primers were designed to hybridize at exon-exon junctions. The *Arg7* cDNA fragment was amplified with an upstream oligonucleotide primer that hybridizes to pKS vector sequences, and thus the endogenous *Arg7* mRNA was not detected. To ensure the specificity of the primers for these transcripts, the PCR products obtained after analysis of RT-PCR were electrophoresed in agarose gels, which revealed a single product from each reaction (Figure 8). As an additional control to ensure that a single PCR product was obtained during real-time PCR, melting curves were determined. At temperatures below the  $T_m$  of a

PCR product, SYBR green bound to the double-stranded DNA gives a fluorescent signal. As the temperature is slowly increased to 95°C, a rapid decrease in fluorescence is detected at the  $T_m$ , when each fragment melts. As  $T_m$  is dependent on the nucleotide content and fragment length, the complexity of PCR products can be determined from such a melting curve. A single PCR fragment is revealed by a single, sharp decrease in fluorescence signal at its specific  $T_m$ . The melting curve analysis of PCR products with primers *Arg1*, *Tub* and *Pdk* demonstrated the formation of one single product and little primer-dimer formation. To emphasize the importance of the melting curve analysis, it was discovered that a pair of primers designed to amplify a fragment from the *psaB* DNA yielded more than one product in PCR amplification and the oligos were not used for further analysis (data not shown).

### **3.6.1 Mathematical analysis of the data**

Real time RT-PCR amplification efficiency was calculated from the rate of fluorescence increase using the Applied Biosystems 7900 software and the Comparative Ct Method (Applied Biosystems). The threshold cycle (Ct) indicates the fractional number of cycles at which the amount of amplified target reaches the midpoint of the amplification (Figure 9). The Ct value is inversely proportional to the log of the initial copy number (Higuchi *et al.*, 1993), and Ct values from amplification containing target template of known concentrations (or known dilutions) are used to establish a standard quantification curve. Therefore, a standard curve is generated by plotting the Ct value against log of the initial

copy number or log of serial dilutions (Figure 10). The slope of the standard curve reflects the DNA replication efficiency of the PCR. The slope of the standard curve reflects the replication efficiency (E) of the PCR. The theoretical efficiency of amplification is equal to 1; a doubling in copy number per round of amplification. In the first cycle of the PCR, the resulting copy number of product ( $C_1$ ) is related to the initial target copy number ( $C_0$ ) by the equation

$$C_1 = C_0(1 + \text{efficiency})$$

The corresponding real time PCR efficiency of one cycle in the exponential phase was calculated from the slope of the standard curve according to the equation:

$$E = [(10^{-1/\text{slope}}) - 1] \times 100$$

When the efficiency is equal to 1, the slope of the standard curve is equal to -3.33 since every 10 fold difference in quantity translates to a difference of 3.33 Cts (ABI prism 7700 SDS user guide, Applied Biosystems, Argaw *et al.*, 2002). For amplicons designed and optimized according to Applied Biosystems guidelines (amplicon size < 150 bp), the efficiency is close to 1. Standard curves were accepted when the value of  $r^2$  (measure of the fit of linear regression) was higher than 0.9 (Figure 10). Values obtained for standard curves are: *Arg1* ( $r^2=0.9997$ ) with a slope value of -3.3786, *Pdk* ( $r^2=0.9879$ ) with a slope value of -3.6545 and *Tub* ( $r^2=0.9941$ ) with a slope value of -3.1344. The efficiency of the standard curves are: 0.99 for *Arg1*, 1 for *Pdk* and 0.94 for *Tub*.

The expression level of the reporter gene transcript was calculated based on the Ct of the sample and expressed relative to that of the reference. Each sample was prepared in duplicate or triplicate. The mean Ct of each reaction

performed with the *Arg1*, *Pdk* and *Tub* oligos was calculated by the SDS 2.0 program.

$$(\text{Ct target} - \text{Ct reference})_{\text{condition 1}} - (\text{Ct target} - \text{Ct reference})_{\text{condition 2}} =$$

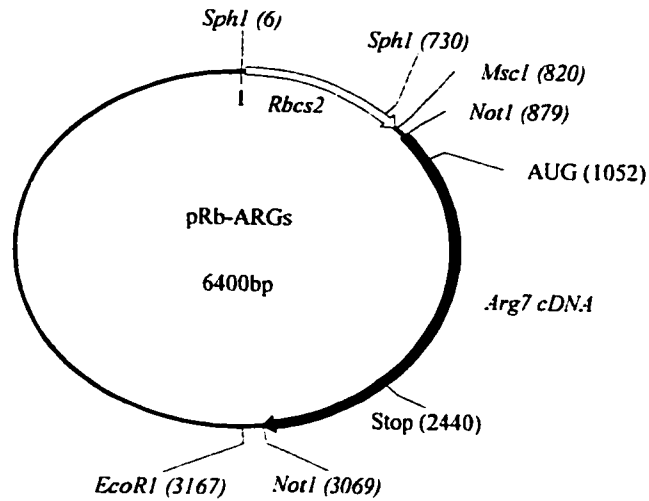
$$(\Delta\text{Ct exposed to metal} - \Delta\text{Ct not exposed to metal}) =$$

$$\text{Fold induction} = 2^{-\Delta\Delta\text{Ct}}$$

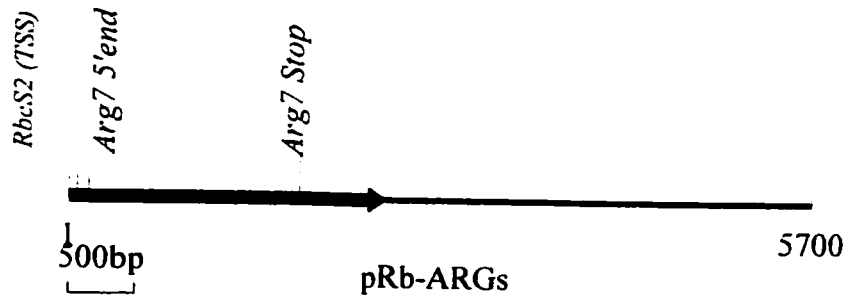
The results of induction were calculated as the fold induction of RNA obtained from culture exposed to metal versus culture not-exposed to metal and the results are presented in Table 4 and Figure 11. There is a direct correlation of *Arg7* cDNA transcript levels and the concentration of either Ni or Cd. Strain Ni70 exposed to 70.5  $\mu\text{M}$  free  $\text{Cd}^{2+}$  results in a  $4.2 \pm 0.3$  fold increase in expression of the reporter gene versus the strain not exposed to metal, when the results were normalized to the *Pdk* internal standard. When the results were normalized to the *Tub* internal standard, a  $14.3 \pm 0.7$  fold induction was obtained (Table 4). Moreover, cultures exposed to 217  $\mu\text{M}$  free  $\text{Ni}^{2+}$  resulted in  $2.3 \pm 0.7$  fold induction versus cultures not treated with metal, when the results were normalized to the *Pdk* internal standard, and  $12.5 \pm 4.3$  fold induction when the results were normalized to the *Tub* internal standard. As the concentration of Cd or Ni is increased the reporter gene transcript level also increases. In addition, it is shown that Ni is a less stronger inducer than Cd (Figure 11). This was also observed (Zenk, 1996) by measuring the concentration of phytochelatins in *R. serpentine* upon induction with Cd and other trace metals.



A)



B)



**Figure 1. Map of pRb-ARGs plasmid (Auchincloss, 1999).**

A) The circular map of the plasmid that contains the *Arg7* cDNA expression cassette used as a reporter gene. Between the *RbcS2* 3'end and the *Arg7* 5' end there is pKS- polylinker derived sequence. B) Linear map of the pRb-ARGs plasmid after digestion with the *Sph I* restriction enzyme. Digestion with *Sph I* left 26 bp upstream the *RbcS2* transcription start site is indicated by *RbcS2* (TTS). The *Arg7* 5' end starts 119 downstream of the *RbcS2* (TTS).

aggcgctgcagcgagttgtggggcatgcgtcatgcgcagacggcccctggacgacaaggcgttgagttggcgtt  
 tggagggtgtgggacgacgtggggtttgtgccgtcaaagcacagaaacagaaggcgtgaccgttttac**Sac 1**  
 atgatgtagcatggattgaataatgacatgtgattttttgttacaagcgacgaatgcgtgggggt**5' end of endogenous Arg7**  
 aggggtttcagtcgcccgattgcgcattgcacacgtgaccaaatttatgctcaacgacgtgaccattgctttata  
 catacttgtgtatcggttggcacttataacaattggctc**5' end of Arg7 cDNA**  
 aagccccagttcaacaagtccgatagccaa**ATG**gccccgctcgctctccagcatcaaggggcctctaagtgcct  
 cgcggaaccacagcgaagtgtgctcgcgttg**3'end of exon 1** **Sal 1**  
 gcaatcgaaa

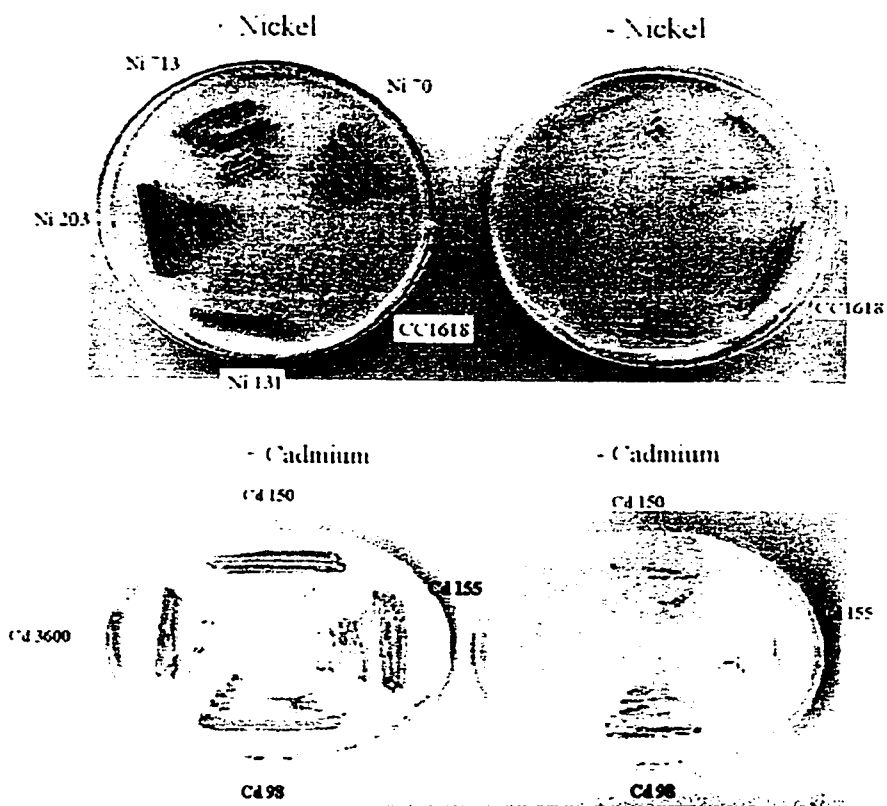
**Figure 2. Arg7 genomic DNA used to prepare the RNase probe.**  
 The genomic sequence of *Arg7* from nucleotide 1801 to 2251 is shown in the figure. The RNase probe was prepared by cloning a 353 *Sac I*-*Sal I* fragment into pBS vector. This vector was cut with *EcoRI* and transcription was done by T3 RNA polymerase.

<b>Name</b>	<b>Genotype</b>	<b>Phenotype</b>
CC1618	<i>Arg7, cw15</i>	arginine auxotroph cell wall mutant
CC425	<i>Arg7, cw15</i>	arginine auxotroph cell wall mutant
CC424	<i>Arg7, cw15</i>	arginine auxotroph cell wall mutant
CC1618P <sup>a</sup>	<i>cw15</i>	cell wall mutant
Beck strain	<i>Pf-14, Arg7</i>	paralyzed flagella arginine auxotroph

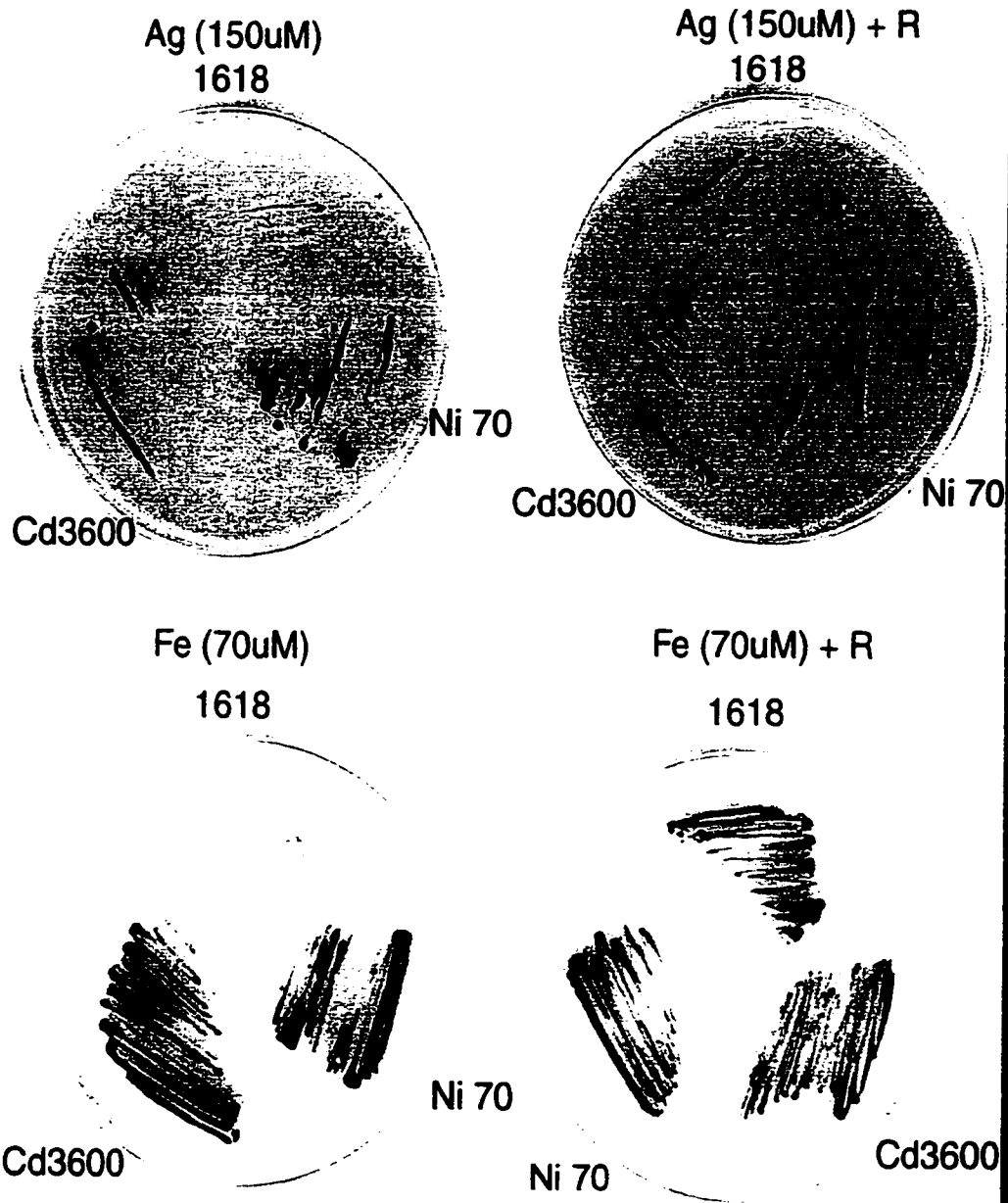
**Table 1. Strains of *C. reinhardtii* used for experiments.**

<b>Name</b>	<b>Genotype</b>	<b>Selection</b>
Ni70	<i>cw15</i>	100 uM nickel
Ni131	<i>cw15</i>	100 uM nickel
Ni203	<i>cw15</i>	100 uM nickel
Ni713	<i>cw15</i>	100 uM nickel
Cd3600	<i>cw15</i>	150 uM cadmium
Cd98	<i>cw15</i>	150 uM cadmium
Cd155	<i>cw15</i>	150 uM cadmium
Cd150	<i>cw15</i>	150 uM cadmium

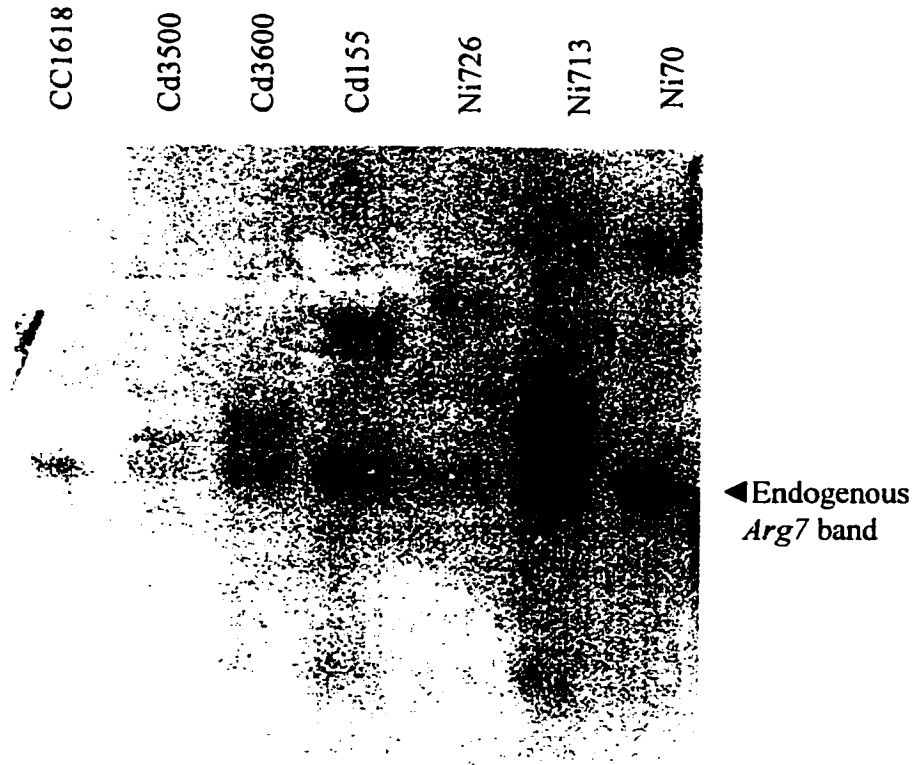
**Table 2. Strains of *C. reinhardtii* mutants obtained after selection on nickel and cadmium. All strains have *Arg7* promoterless gene integrated in the genome and grow only in presence of metal. Cell wall mutants (*cw15*).**



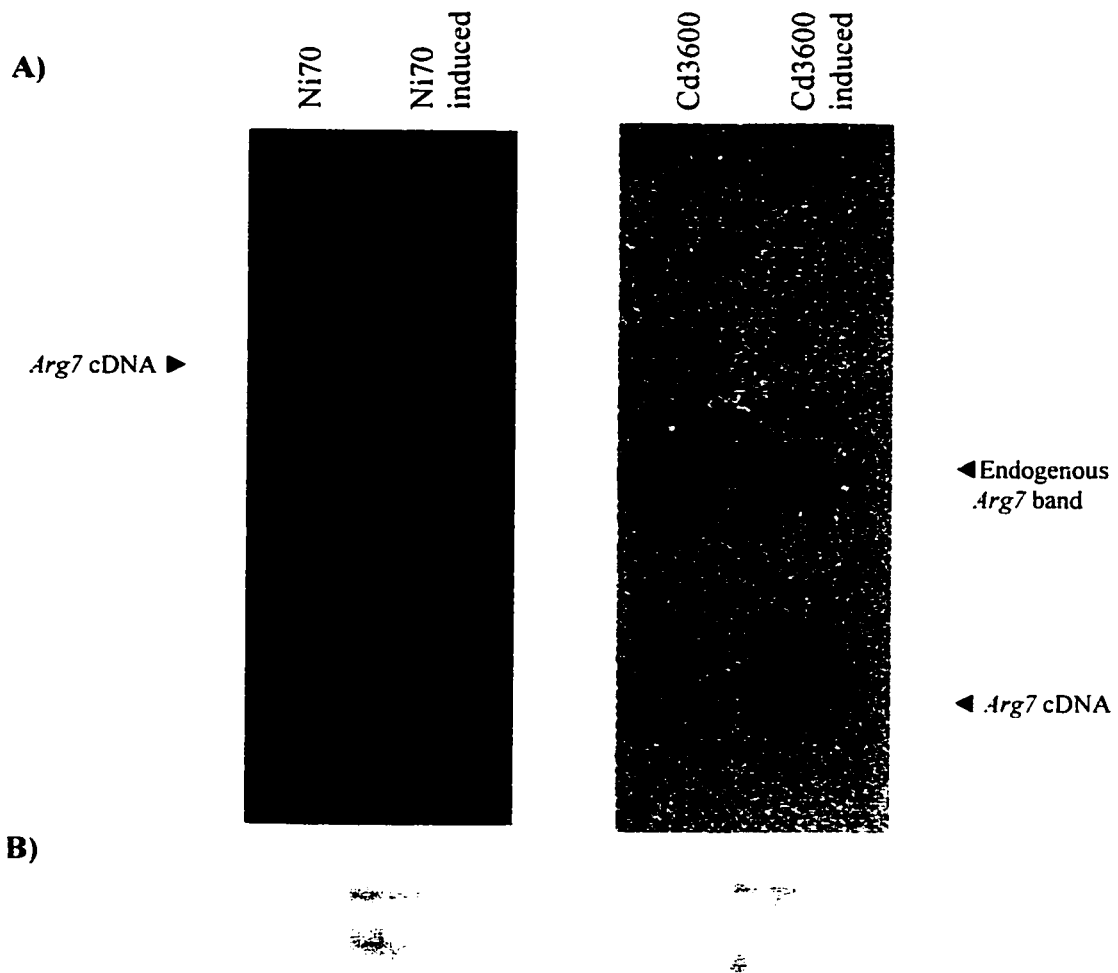
**Figure 3. *C. reinhardtii* transformants strains selected on metal.** Ni70, Ni113, Ni203, Ni713 were selected on nickel and strains Cd98, Cd150, Cd155 and Cd3600 were selected on cadmium.



**Figure 4. *C. reinhardtii* transformant strains grow in 70 uM iron and 150 uM silver.** The plates supplied with arginine were control plates to verify that the concentration of the metal was not lethal. CC1618 is the control strain and does not grow in presence of metals if not supplied with arginine.

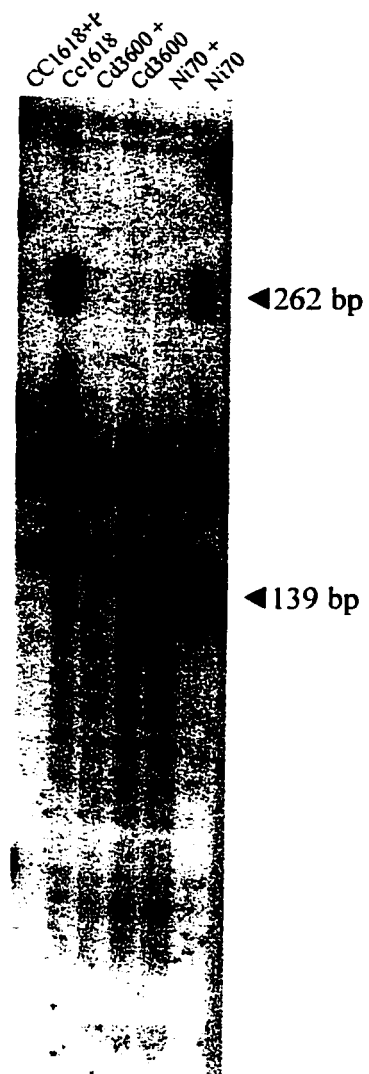


**Figure 5. Southern analysis of DNA from transformants.** Gel blot of total DNA digested with *Apal* and *SacI* was used for hybridization. Numbers above the line correspond to individual transformants. The blot was hybridized with a 5.7 kb cDNA *Arg7* fragment (Material and Methods). Additional copies are visible as extra bands. Strain Cd3500 and strain Ni726 are  $arg^+$  even on plates lacking metal.



**Figure 6. Northern blot analysis of transformants.**

A) RNA blots were hybridized to an *ARG7* probe as described in Material and Methods. The band corresponding to endogenous *Arg7* gene is present in control strain CC1618 (not shown) and in the induced and non induced NI70 and Cd3600 strains. The extra bands corresponding to inserted *Arg7* gene is present only in the exposed strains as indicated by arrows. B) The RNA filters were stained with methylene blue solution to ensure equal gel loading.

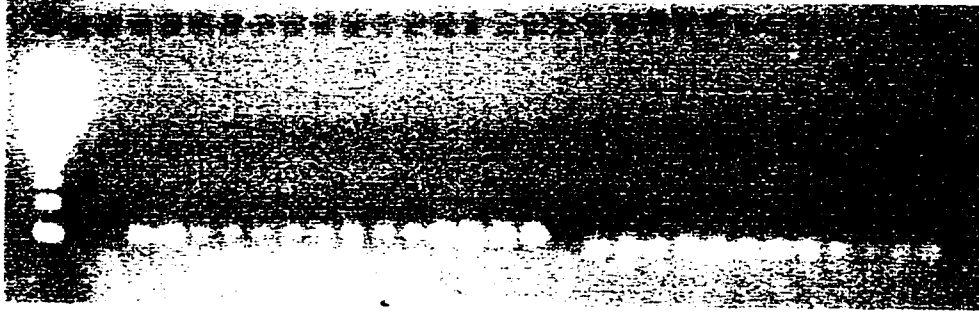


**Figure 7. RNase protection assay was performed on transformants exposed and non exposed to metal. Lanes 1-6 contain poly-(A)<sup>+</sup> RNA. The + symbol indicate strains exposed to metal. Control strain for endogenous *Arg7* is strain CC1618 and for inserted *Arg7* is strain CC1618+P.**



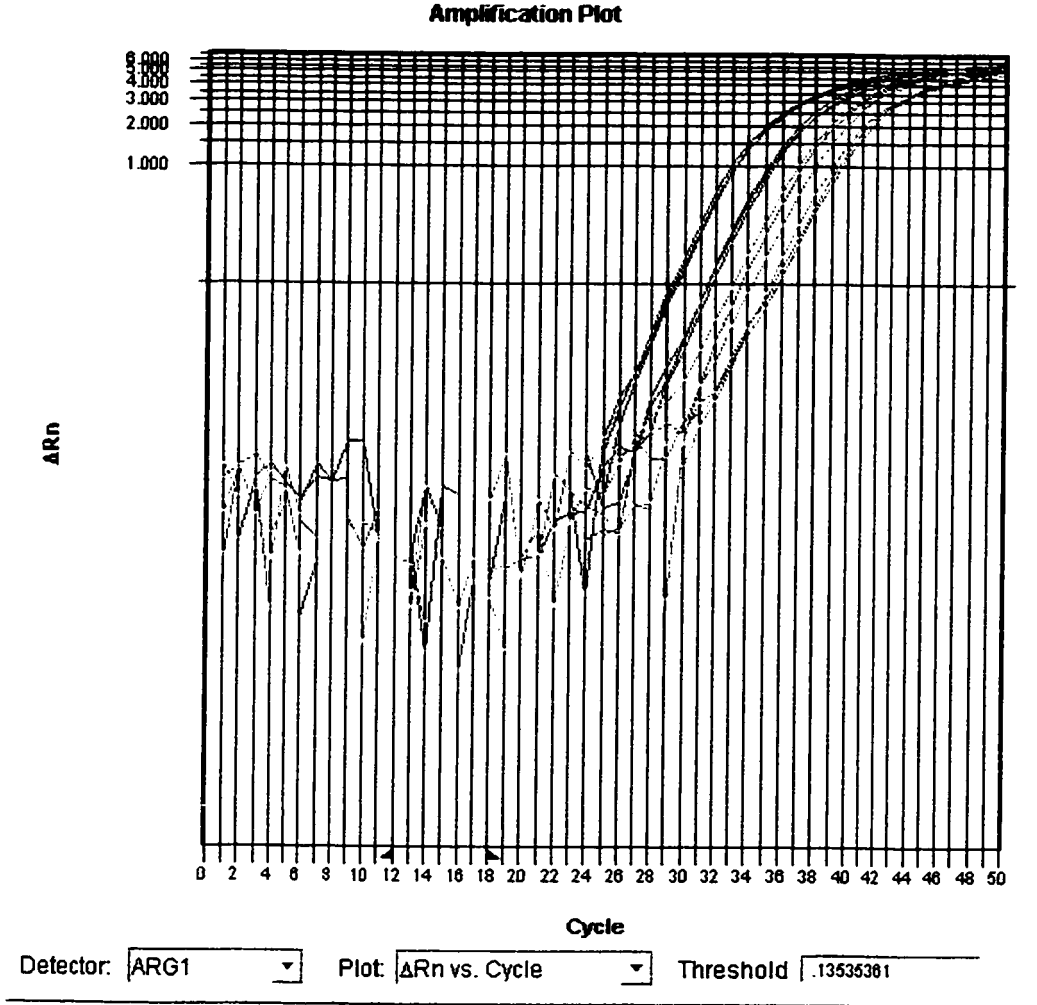
*Argl* oligos

*Pdk* oligos



**Figure 8. Example of RT-PCR amplified fragment.** After PCR amplification the samples were run in a 2% agarose gel to verify the size of the amplified fragments and the specificity of the oligos.

Amplification Plot

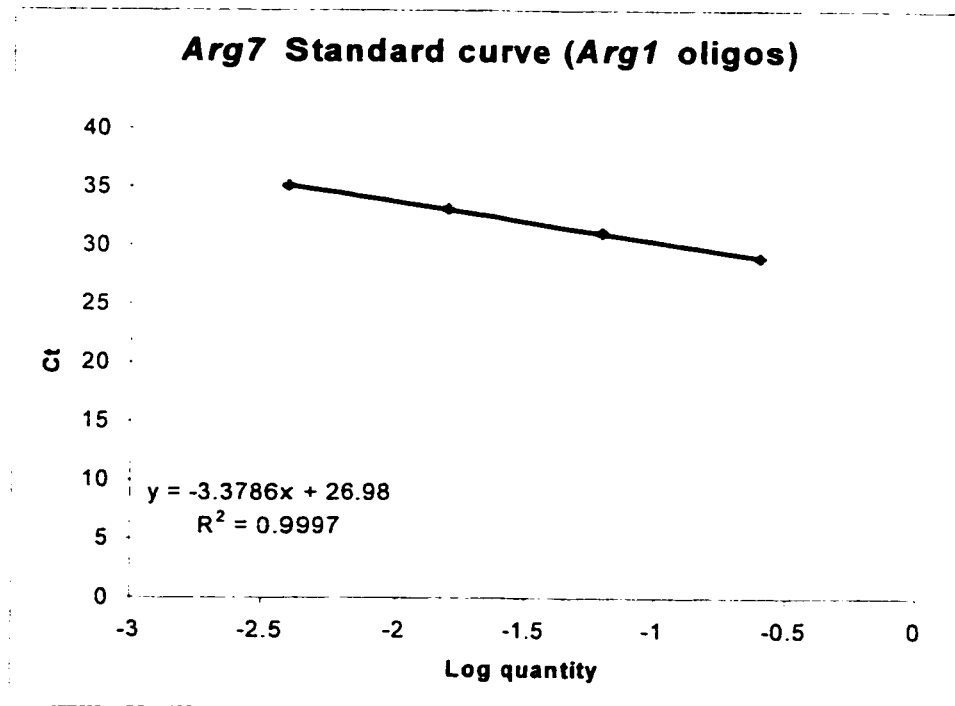


**Figure 9. Example plot of RT-PCR product during amplification.** PCR was performed with *Arg1* oligos on RNA from Ni70 that had been exposed to 10 uM cadmium. The samples were prepared in triplicate. The curves in dark blue that appear on the left side are from the 4-fold dilutions of the RT reaction. Light blue curves are triplicate amplifications of the 16-fold dilution, green blue curves are from 64-fold dilution and green curves are from 256 fold dilutions of the RT reaction. The red line indicates the threshold level of fluorescence from which Cts are derived.

A)

Dilution values total RNA	Log dilution values total RNA	<i>Arg1</i> average Ct
1/4	-0.60	28.9
1/16	-1.20	31.1
1/64	-1.81	33.1
1/256	-2.41	35.1

B)



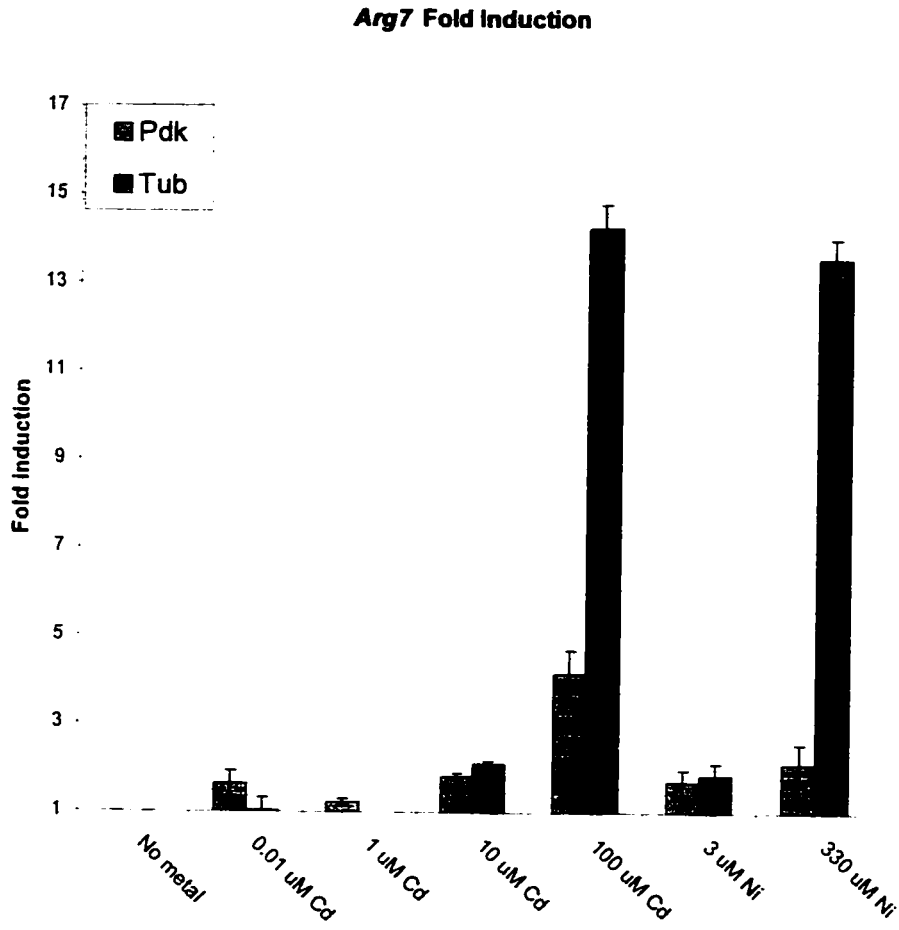
**Figure 10. Cts obtained from RT-PCR amplification and standard curve plot.** A) Values of Ct for standard curve for obtained using *Arg1* pair of oligos. B) Standard curve plot. The efficiency of amplification (E) can be calculated from the slope of the standard curve.

<b>Primer</b>	<b>Sequence</b>	<b>Application</b>
<i>Arg1F</i>	ACGTGCGTCGACCACTCTAGA	5' primer for the inserted <i>Arg7</i> cDNA gene
<i>Arg1R</i>	GCCTCGCGTCAATTTGACA	3' primer for the inserted <i>Arg7</i> cDNA gene
<i>Pdk 570 F</i>	ACATCAAAAAGGTGTACAACCAGTACTAC	5' primer <i>Pdk</i>
<i>Pdk 720 R</i>	GTGGCCAGAGAGTCCAGCAT	3' primer <i>Pdk</i>
<i>PsaB 250F</i>	TTTGCTGGTTGGCTTCACTT	5' primer <i>PsaB</i>
<i>PsaB 300R</i>	TCAAAGATGCTGAATCTCGTTTAAA	3' primer <i>PsaB</i>
<i>Tub 1567 F</i>	GCGGTTGACAAGCTGTACA	5' primer <i>Tub</i>
<i>Tub 1635 R</i>	CCGCCCGGATCCATGT	5' primer <i>Tub</i>

**Table 3. Oligonucleotide primer sequences.** Primer sequences used for RT-PCR amplification. Forward primer (F) and reverse primer (R).

Strain	[metal]	[free metal] <sup>a</sup>	RT trial	PCR trial	<i>Arg7</i> reporter <i>Pdk</i> <sup>b</sup>	<i>Arg7</i> reporter <i>Tub</i> <sup>c</sup>
Ni70	None added		1	1	1	1
			2	2	1	1
			2	3	1	1
					<b>X=1.0</b>	<b>X=1.0</b>
	0.01 uM Cd	0.007 uM Cd	1	1	1.42	0.85
			2	2	2.04	1.15
			2	3	1.44	1.09
					<b>X=1.6±0.2</b>	<b>X=0.99±0.2</b>
	1 uM Cd	0.7 uM Cd	1	1	1.34	0.78
			2	2	1.18	0.88
			2	3	1.16	0.98
					<b>X=1.3±0.1</b>	<b>X= 0.86±0.1</b>
	10 uM Cd	7.04 uM Cd	1	1	1.75	1.92
			2	2	1.79	2.03
			2	3	1.91	2.35
				<b>X=1.8±0.1</b>	<b>X= 2.1±0.2</b>	
100 uM Cd	70.5 uM Cd	1	1	4.45	13.53	
		2	2	4.60	13.52	
		2	3	3.40	15.77	
				<b>X=4.2±0.3</b>	<b>X= 14±0.7</b>	
Ni70	3 uM Ni	2.11 uM Ni	1	1	1.32	1.92
			2	2	1.98	1.82
			2	3	1.78	1.80
					<b>X=1.7±0.4</b>	<b>X=1.87±0.1</b>
	330 uM Ni	217 uM Ni	1	1	2.75	9.48
			2	2	1.73	14.45
2			3	1.84	16.61	
				<b>X=2.3±0.7</b>	<b>X=12.5±4.3</b>	
CC1618	None added		1	1	0.01	0.04
			2	2	0.04	0.13
			2	3	0.05	0.13
CC1618+P	None added		1	1	50.41	38.55
			2	2	21.67	35.63
			2	3	19.12	37.34

**Table 4. Values of fold induction of strain Ni70 exposed to metal relative to non exposed strain.** <sup>a</sup> The free metal concentration that the cultures were exposed was calculated using the Visual MINTEQ ver.2.11. This version was completed by Jon Peter Gustafsson, KTH, Dept. of Land and Water Resources Engineering, Stockholm, Sweden. <sup>b, c</sup> The *Arg7* gene transcript level was normalized versus the *Pdk* and *Tub* (internal standards) transcript level.



**Figure 11. Graphical representation of *Arg7* reporter gene induction upon metal exposure.** The real time RT-PCR was performed three times. Two times in on RT preparation and once in another RT preparation. Error bars indicate the variability in between the three trials.

## CHAPTER 4. DISCUSSION

The long-term goal of this project is the identification and characterization of metal tolerance mechanisms and their regulation in the eukaryotic green alga *C. reinhardtii*. Towards this goal, a new promoter-trapping screen was employed to identify genes that are induced by exposure to toxic metals. A reporter gene that originates from *C. reinhardtii* was used because heterologous genes are poorly expressed in this alga (Stevens *et al.*, 1996). The reporter gene is an *Arg7* cDNA minigene cassette, which complements the *arg<sup>-</sup>* phenotype of *arg7* mutations restoring arginine prototrophy (Auchincloss *et al.*, 1999). Another advantage of this system is that the *Arg7* cDNA minigene is used both as a selectable marker for transformation *and* as the reporter gene. Thus, all transformants must express the reporter gene, thereby allowing the screening only of transformants with potentially regulated cDNA insertions. The importance of this feature is emphasized by limitations of the only established promoter trap system for *C. reinhardtii* (Haring and Beck, 1997) in which less than 3% of transformants with random insertions of the promoterless *Rsp3* gene express this gene and could potentially identify a differentially regulated gene at the insertion site.

A possible limitation of this method is that insertions into genes that are *required* for metal tolerance can only be identified by reporter gene insertions that do not eliminate their function. As transcriptional regulatory elements can often act over several kilobases, it is possible that many insertions can come under the regulation of metal promoters, without disrupting the genes. One possible

solution would be to perform this screen in a diploid strain. Disruption of a metal-regulated gene, which is required for metal tolerance, would be lethal in the presence of metal only when two wild-type copies are required (i.e. when the gene is haplo-insufficient).

This study addressed the effects of cadmium and nickel. Cadmium is a toxic metal which activates the defense system of the cell even in concentrations as low as 1 pM to 25 uM (Siripornadulis *et al.*, 2002, Ahner *et al.*, 1994). Nickel is less toxic and is even required as a cofactor by certain enzymes. However this metal is an important environmental pollutant, particularly in Canada due to the major nickel mining industry. Moreover, inclusion of Ni in these experiments might reveal a novel nickel defense system or that Ni tolerance is mediated by known metal detoxification systems (e.g. phytochelatins).

From the eight candidates having a metal-regulated reporter gene, strains Ni70 and Cd3600 were extensively studied because of their near wild-type growth rate on media with metal and nearly complete inability to grow in the absence of metal. Southern analysis used to investigate the nature of the integration events in these strains revealed that Ni70 and Cd3600 have one insertion of the reporter gene (Figure 3). Northern analysis of the strains revealed that in Ni70 and Cd3600 the *Arg7* cDNA reporter gene is induced at the level of transcription, mRNA stability or both, by either Cd (Cd3600) or Ni (Ni70) (Figure 4). However, Northern analysis of metal-exposed strains was not very reproducible and insufficiently sensitive even when performed with poly-(A)<sup>+</sup> RNA.



In an effort to increase the sensitivity of detection, an RNase protection assay was developed (Figure 5). Although RNase protection is a more sensitive and quantitative assay of RNA abundance than is the Northern blot, the attempt to detect the reporter gene transcript was unsuccessful. Our inability to detect the transcript in the promoter-trapped transformants could be due to low transcript abundance, heterogeneity of the 5' end, or both factors. The latter might be expected for artificial fusions of a promoter to the inserted reporter gene. The inability to detect induction of the *Arg7* cDNA by metal in these experiments could also have resulted from metal chelation by citrate in the medium (see Results and the discussion below).

The final objective of the current project was to quantify the level of the reporter gene transcript and determine if it correlates with the metal concentration exposed to the strain. In addition to the poor sensitivity of the Northern blot and RNase protection assays, these methods were cumbersome and required large quantities of purified poly-(A)<sup>+</sup> mRNA. A real time RT-PCR was then developed to analyze the level of expression of *Arg7* cDNA in total RNA samples, using Ni70.

During the analysis of RT-PCR we realized that another important factor for the non-reproducibility of results with all three assays could be EDTA in the “old” TAP medium used for induction. The exposure of strain Ni70 to metal was performed in media completely lacking chelating agent and trace metals, thus avoiding the detoxification of added metal that these components could create. Although the medium in which strains Ni70 and Cd3600 were exposed to metal

prior to Northern blot analyses contains citrate instead of EDTA, the citrate is still a complexing agent and a small difference in its quantity can shift the equilibrium from free metal ions towards chelated metal ions. It is possible that the amount of free ions available for induction was not the one calculated due to composition of the medium.

The real time RT-PCR assay was developed to quantify the level of the *Arg7* cDNA transcript; primers were designed to bind to the inserted reporter gene only. This method is sensitive and can be performed on samples of total RNA. It is also better suited to the analysis of multiple samples, which are contained in 96-well plates. Quantification of the cDNA transcript was determined relative to two control cellular mRNAs: *Pdk* and *Tub*. The results of the RT-PCR are presented in Table 3. The experiment was repeated twice with one cDNA preparation and once with a second cDNA preparation for a total of three trials. The results show a direct correlation of the concentration of the metal exposed to the culture and the level of *Arg7* cDNA transcript (Table 4). Exposure of strain Ni70 to increasing concentrations of metals resulted in an increase of the reporter gene transcript. Although, the levels of induction normalized to the *Pdk* and *Tub* genes are not similar they follow the same trend. Previous studies have revealed that the normalization with the mRNA from a single gene can lead to relatively large errors in the sample (Vandesompele *et al.*, 2002). These are encouraging results and future assays should be normalized with other control genes to determine which level of induction is more probable.

In *C. reinhardtii* few genes have been studied for differential expression under specific conditions and it was demonstrated that the level of induction varies. Transcriptional responses to 25  $\mu\text{M}$  Cd were analyzed by mRNA differential display (Rubinelli *et al.*, 2002). Most of the transcripts identified (coding for both chloroplast and nuclear genes) had an induction varying between 0.5- and 6-fold, although one transcript was induced by 20-fold. Low- $\text{CO}_2$  conditions induce a gene encoding alpha-ketoglutarate aminotransferase; the mRNA increased 4- to 5-fold 4 h after cells were switched from high- $\text{CO}_2$  to low- $\text{CO}_2$  growth conditions (Chen *et al.*, 1996). The *Gsa* gene, encoding the chlorophyll biosynthetic enzyme, glutamate 1-semialdehyde aminotransferase, is light-regulated. The level of mRNA nearly doubled during the first 0.5 h in the light and increased over 26 fold after 2 h in the light (Matters and Beale, 1994). These examples demonstrate that the level of gene induction varies greatly and various factors can induce more than one gene. The results of the real time RT-PCR assays are encouraging because induction of the *Arg7* cDNA transcript was observed and this induction correlates with an increase in the concentration of Cd and Ni.

### **Future Directions**

Now that we have a reliable real time RT-PCR assay for quantification of the expression of the *Arg7* cDNA, this method should be used to determine whether similar metal induction occurs in the other strains obtained, particularly Cd3600. It would also be important to determine whether the insertions affect

metal tolerance, as they could mutate or disrupt genes encoding such functions. Performing the screen in an *arg7* homozygous mutant diploid strain, which was recently generated in our laboratory, might reveal a higher percentage of metal regulated insertions.

A long-term goal is to clone and characterize the candidate genes obtained in this study and determine whether their accumulation of mRNA is induced by metal exposure. It is presumed and hoped that the gene at the insertion site is positively regulated by metal exposure and is part of the function in the metal tolerance or detoxification pathway. This may reveal new mechanisms of trace metal toxicity and tolerance, signal transduction pathways and factors that regulate the expression of these tolerance systems.

The final goal is to develop simple assays for bioavailable trace metal ions at environmentally relevant concentrations. If there is a linear correlation between the free concentration of metals tested and the transcript level, a standard curve can be set up and strain Ni70 would be used as a bioassay for the free quantity of metal present in the tested media. This response could be incorporated into a bioassay for monitoring natural soils and waters specifically for bioavailable species of metals. It may then lead to a marketable product for industry and governmental agencies.

## LITERATURE CITED

- Ahner, B. A., Price, N. M. and Morel, M. M. (1994) Phytochelatin production by marine phytoplankton at low free metal ion concentrations: Laboratory studies and field data from Massachusetts Bay. *Proc. Natl. Acad. Sci. USA* **91**, 8433-8436
- Al-Laham, A., Rhode, V., Heim, P., Leuchter, R., Veeck, J., Wunderlich, C., Wolf, K. and Zimmerman, M. (1999) Biosynthesis of phytochelatins in the fission yeast. Phytochelatin synthesis: a second role for the glutathione synthetase in *Schizosaccharomyces pombe*. *Yeast* **15**, 385-396
- Andrew, J., Smith, C., Kramer, U. and Baker, J.M. (1995) Role of metal transport and chelation in nickel hyperaccumulation in the genus *Alyssum*. Fourteenth Annual Symposium, University of Missouri-Columbia. Current topics in plant biochemistry, physiology and molecular biology, April 19-22, pp. 9-10
- Argaw T., Ritzhaupt A. and Wilson C.A. (2002) Development of a real time quantitative PCR assay for detection of porcine endogenous retrovirus. *J Virol Methods*. **106**, 97-106
- Auchincloss A.H., Loroach A.I., Rochaix J.D. (1999) The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: cloning of the cDNA and its characterization as a selectable shuttle marker. *Mol Gen Genet*. **261**, 21-30
- Beyersmann, D. and Hechtenberg, S. (1997) Cadmium, gene regulation, and cellular signalling in mammalian cells. *Toxicol. Appl. Pharmacol.* **144**, 247-261
- Bruins, M.R., Kapil, S. and Oehme, F.W. (2000) Microbial resistance to metals in the environment. *Ecotoxicol. Environ. Saf.* **45**, 198-207
- Cerutti H., Johnson A.M., Gillham N.W., Boynton J.E. (1997) A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. *Genetics*. **145**, 97-110
- Chardonens, A.N., Koevoets, P.L., van Zanten, A., Schat, H. and Verkleij, J.A. (1999) Properties of enhanced tonoplast zinc transport in naturally selected zinc-tolerant *Silene vulgaris*. *Plant Physiol.* **120**, 779-786
- Chen Z.Y., Burow M.D., Mason C.B., Moroney J.V. (1996) A low-CO<sub>2</sub>-inducible gene encoding an alanine: alpha-ketoglutarate aminotransferase in *Chlamydomonas reinhardtii*. *Plant Physiol.* **112**, 677-84
- Ciriolo, M.R., Civitareale, P., Carri, M.T., De Martino, A., Galiazzo, F. and Rotilio, G. (1994) Purification and characterization of Ag,Zn-superoxide dismutase from *Saccharomyces cerevisiae* exposed to silver. *J. Biol. Chem.* **269**, 25783-25787

- Clemens, S., Antosiewicz, D.M., Ward, J.M., Schachtman, D.P. and Schroeder, J.I. (1998) The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast. *Proc. Natl. Acad. Sci. USA* **95**, 12043-12048
- Clemens, S., Kim, E.J., Neumann, D. and Schroeder, J.I. (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J.* **18**, 3325-3333
- Cobbett, C. (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. *Curr. Opin. Plant Biol.* **3**, 211-216
- Cobbett, C. and Goldsbrough, P. (2002) Phytochelatin and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* **53**, 159-182
- Debuchy R., Purton S. and Rochaix J.D. (1989) The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. *EMBO J.* **8**, 2803-9
- Desi I., Nagymajtenyi L. and Schulz H. (1998) Behavioural and neurotoxicological changes caused by cadmium treatment of rats during development. *J Appl Toxicol.* **18**, 63-70
- Fischer, N. and Rochaix, J.D. (1999) The flanking regions of *PsaD* drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii*. *Mol. Genet. Genomics* **265**, 888-894
- Freeman W.M., Walker S.J. and Vrana K.E. (1999) Quantitative RT-PCR: pitfalls and potential. *Biotechniques.* **26**, 112-22
- Fortin, C. and Campbell, P.G. (2001) Thiosulfate enhances silver uptake by a green alga: role of anion transporters in metal uptake. *Environ. Sci. Technol.* **35**, 2214-2218
- Franklin, S., Ngo, B., Efuat, E. and Mayfield, S.P. (2002) Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *Plant J.* **30**, 733-744
- Goldschmidt-Clermont, M. (1991) Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker of site-directed transformation of *Chlamydomonas*. *Nucleic Acids Res.* **19**, 4083-4089
- Gralla, E.B., Thiele, D.J., Silar, P. and Valentine, J.S. (1991) ACE1, a copper-dependent transcription factor, activates expression of the yeast copper, zinc superoxide dismutase gene. *Proc. Natl. Acad. Sci. USA* **88**, 8558-8562

- Grill, E., Winnacker, E.L. and Zenk, M.H. (1991) Phytochelatins. *Methods Enzymol.* **205**, 333-341
- Ha, S.B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B. and Cobbett, C.S. (1999) Phytochelatin synthase genes from *Arabidopsis* and the yeast *Schizosaccharomyces pombe*. *Plant Cell* **11**, 1153-1164
- Hall, J.L. (2002) Cellular mechanism for heavy metal detoxification and tolerance. *J. Exp. Bot.* **53**, 1-11
- Haring, M. and Beck, C. (1997) A promoter trap for *Chlamydomonas reinhardtii*: development of a gene cloning method using 5'RACE-based probes. *Plant J.* **11**, 1341-1348
- Hill, K., Hassett, R., Kosman, D. and Merchant, S. (1996) Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability. *Plant Physiol.* **112**, 697-704
- Higuchi, R., C. Fockler, G. Dollinger, and R. Watson. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Bio/Technology* **11**, 1026-1030.
- de Hostos E.L., Togasaki R.K., and Grossman A. (1988) Purification and biosynthesis of a derepressible periplasmic arylsulfatase from *Chlamydomonas reinhardtii*. *J Cell Biol.* **106**, 29-37
- Howe, G. and Merchant, S. (1992) Heavy metal-activated synthesis of peptides in *Chlamydomonas reinhardtii*. *Plant Physiol.* **98**, 127-136
- Irihimovitch, V. and Shapira, M. (2000) Glutathione redox potential modulated by reactive oxygen species regulates translation of Rubisco large subunit in the chloroplast. *J. Biol. Chem.* **275**, 16289-16295
- Kang, Y. and Mitchell, D.R. (1998) An intronic enhancer is required for deflagellation-induced transcriptional regulation of a *Chlamydomonas reinhardtii* dynein gene. *Mol. Biol. Cell* **9**, 3085-3094
- Klapheck, S., Fliegner, W. and Zimmer, I. (1994) Hydroxymethyl-phytochelatins [(gamma-glutamylcysteine)*n*-serine] are metal-induced peptides of the Poaceae. *Plant Physiol.* **104**, 1325-1332
- Kubota, H., Sato, K., Yamada, T. and Maitani, T. (2000) Phytochelatin homologs induced in hairy roots of horseradish. *Phytochemistry* **53**, 239-245

- Leisinger, U., Rufenacht, K., Fischer, B., Pesaro, M., Spengler, A., Zehnder, A.J. and Eggen, R.I. (2001) The glutathione peroxidase homologous gene from *Chlamydomonas reinhardtii* is transcriptionally up-regulated by singlet oxygen. *Plant Mol. Biol.* **46**, 395-408
- Lemaire, S., Keryer, E., Stein, M., Schepens, I. I., Issakidis-Bourguet, E., Gérard-Hirne, C., Miginiac-Maslow, M. and Jacquot, J.P. (1999) Heavy-metal regulation of thioredoxin gene expression in *Chlamydomonas reinhardtii*. *Plant Physiol.* **120**, 773-778
- Liu, P., Goh, C.J., Loh, C.S. and Pua, E.C. (2002) Differential expression and characterization of three metallothionein-like genes in Cavendish banana (*Musa acuminata*). *Physiol. Plant.* **114**, 241-250
- Loppes, R. and Radoux, M. (2001) Identification of short promoter regions involved in the transcriptional expression of the nitrate reductase gene in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **45**, 215-227
- Lu, Y.P., Li, Z.S. and Rea, P.A. (1997) *AtMRP1* gene of *Arabidopsis* encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. *Proc. Natl. Acad. Sci. USA* **94**, 8243-8248
- Macfie, S.M. and Welbourn, P.M. (2000) The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). *Arch. Environ. Contam. Toxicol.* **39**, 413-419
- Marjorette, M., Pena, O., Koch, A. K. and Thiele, D. (1998) Dynamic regulation of copper uptake and detoxification genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18**, 2514-2523
- Matters G.L., and Beale S.I. (1994) Structure and light-regulated expression of the *gsa* gene encoding the chlorophyll biosynthetic enzyme, glutamate 1-semialdehyde aminotransferase, in *Chlamydomonas reinhardtii*. *Plant Mol Biol.* **24**, 617-29
- Matz M.V. and Lukyanov S. A.(1998) Differential strategies of differential display: areas of application. *Nucleic Acid Research.* **26**, 5537-5543
- Meijer H.J., Arisz S.A., Van Himbergen J.A., Musgrave A., and Munnik T. (2001) Hyperosmotic stress rapidly generates lyso-phosphatidic acid in *Chlamydomonas*. *Plant J.* **25**, 541-8
- Menke U. and Mueller-Roeber B. (2001) RNA Fingerprinting of Specific Plant Cell Types: Adaptation to Plants and Optimization of RNA Arbitrarily Primed PCR (RAP-PCR). *Plant Molecular Biology Reporter.* **19**, 33-48



- Minko, I., Holloway, S.P., Nikaido, S., Carter, M., Odom, O.W., Johnson, C.H. and Herrin, D.L. (1999) *Renilla luciferase* as a vital reporter for chloroplast gene expression in *Chlamydomonas*. *Mol. Gen. Genet.* **262**, 421-425
- Morris, C.A., Nicolaus, B., Sampson, V., Harwood, J.L. and Kille, P. (1999) Identification and characterization of a recombinant metallothionein protein from a marine alga, *Fucus vesiculosus*. *Biochem. J.* **338**, 553-560
- Neumann, D. and zur Nieden, U. (2001) Silicon and heavy metal tolerance of higher plants. *Phytochemistry* **56**, 685-692
- Nies D.H. (1992) CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (czc system) in *Alcaligenes eutrophus*. *J Bacteriol.* **174**, 8102-10
- Nishimura, K., Igarashi, K. and Kakinuma, Y. (1998) Proton gradient-driven nickel uptake by vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *J. Bacteriol.* **180**, 1962-1964
- Nozaki H., Misawa K., Kajita T., Kato M., Nohara S. and Watanabe M.M. (2000) Origin and evolution of the colonial volvocales (*Chlorophyceae*) as inferred from multiple, chloroplast gene sequences. *Mol. Phylogenet. Evol.* **17**, 256-268
- Ortiz, D.F., Ruscitti, T., McCue, K.F. and Ow, D.W. (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC vacuolar membrane protein. *J. Biol. Chem.* **270**, 4721-4728
- Pence, N.S., Larsen, P.B., Ebbs, S.D., Letham, D.L., Lasat, M.M., Garvin, D.F., Eide, D. and Kochian, L.V. (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proc. Natl. Acad. Sci. USA* **97**, 4956-4960
- Periz, G. and Keller, L. (1997) DNA elements regulating  $\alpha$ 1-tubulin gene induction during regeneration of eukaryotic flagella. *Mol. Cell. Biol.* **17**, 3858-3866
- Rae, T.D., Schmidt, P.J., Pufahl, R.A., Culotta, V.C. and O'Halloran, T.V. (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805-808
- Rausser, W.E. (1995) Phytochelatins and related peptides: structure, biosynthesis, and function. *Plant Physiol.* **109**, 1141-1149
- Rausser, W.E. and Meuwly, P. (1995) Retention of cadmium in roots of maize seedlings. Role of complexation by phytochelatins and related thiol peptides.

*Plant Physiol.* **109**, 195-202

Robinson, N.J., Tommey, A.M., Kuske, C. and Jackson, P.J. (1993) Plant metallothioneins. *Biochem. J.* **295**, 1-10

Rochaix J.D. (1995) *Chlamydomonas reinhardtii* as the photosynthetic yeast. *Annu Rev Genet.* **29**, 209-30

Romero-Puertas, M.C., McCarthy, I., Sandalio, L.M., Palma, J.M., Corpas, F.J., Gomez, M. and del Rio, L.A. (1999) Cadmium toxicity and oxidative metabolism of pea leaf peroxisomes. *Free Radic. Res.* **31**, S25-S31

Rubinelli, P., Siripornadulsil, S., Gao-Rubinelli, F. and Sayre, R.T. (2002) Cadmium- and iron-stress-inducible gene expression in the green alga *Chlamydomonas reinhardtii*: evidence for H43 protein function in iron assimilation. *Planta* **215**, 1-13

Salnikow, K., An, W.G., Melillo, G., Blagosklonny, M.V. and Costa, M. (1999) Nickel-induced transformation shifts the balance between HIF-1 and p53 transcription factors. *Carcinogenesis* **20**, 1819-1823

Salt, D.E. and Wagner, G.J. (1993) Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a  $\text{Cd}^{2+}/\text{H}^{+}$  antiport activity. *J. Biol. Chem.* **268**, 12297-12302

Schutzendubel A. and Polle A. (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J Exp Bot.* **53**. 1351-65

Siripornadulsil, S., Traina, S., Verma, D.P. and Sayre, R.T. (2002) Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell* **14**, 2837-2847

Slaveykova, V. and Wilkinson, K. (2002) Physicochemical aspects of lead bioaccumulation by *Chlorella vulgaris*. *Environ. Sci. Technol.* **36**, 969-975

Schroda M., Vallon O., Wollman F.A. and Beck C.F. (1999) A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition. *Plant Cell.* **11**, 1165-78

Sodeinde O.A. and Kindle K.L., (1993) Homologous recombination in the nuclear genome of *Chlamydomonas reinhardtii*. *Proc Natl Acad Sci U S A.* **90**, 9199-203

Steffens, J.C. (1990) The heavy metal-binding peptides of plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 553-575

Steven, P., Beate, R. and Ruedi, A. (2000) Measuring gene expression by

- quantitative proteome analysis. *Curr. Opin. Biotech.* **11**, 396–401
- Stiborova, M., Doubravova, M. and Leblova, S. (1986) Comparative study of the effects of heavy metal ions on ribulose-1,5-bisphosphate carboxylase. *Biochem. Physiol. Pflanz.* **181**, 373-379
- Stobart, A.K., Griffiths, W.T., Ameen-Bukhari, I. and Sherwood, R.P. (1985) The effect of Cd<sup>2+</sup> on the biosynthesis of chlorophyll in leaves of barley. *Physiol. Plant.* **63**, 293-298
- Stolz J.,F., Basu P., Oremland R.S. (2002) Microbial transformation of elements: the case of arsenic and selenium. *Int Microbiol* **5**, 201-7
- Sumper, M. and Hallmann A. (1998) Biochemistry of the extracellular matrix of *Volvox*. *Int. Rev. Cytol.* **180**, 51-85
- Tessier, A. and Turner, D.R. (1995) Metal speciation and bioavailability in aquatic systems. John Wiley and Sons, Chichester, New York
- Thomine, S., Wang, R., Ward, J.M., Crawford, M.N. and Schroeder, J. (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to *Nramp* genes. *Proc. Natl. Acad. Sci. USA* **97**, 4991-4996
- Vatamaniuk, O.K., Mari, S., Lu, Y.P. and Rea, P.A. (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. *J. Biol. Chem.* **275**, 31451-31459
- Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **18**, 3-7
- Vatamaniuk, O.K., Mari, S., Lu, Y.P. and Rea, P.A. (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. *Proc. Natl. Acad. Sci. USA* **96**, 7110-7115
- Wemmie, J.A., Wu, A.L., Harshman, K.D., Parker, C.S. and Moye-Rowley, W.S. (1994) Transcriptional activation mediated by the yeast AP-1 protein is required for normal cadmium tolerance. *J. Biol. Chem.* **269**, 14690-14697
- Zenk, M. (1996) Heavy metal detoxification in higher plants. *Gene* **179**, 21-30
- Zhu Z., and Thiele D.J. (1996) Toxic metal-responsive gene transcription. *EXS.* **77**, 307-20