Development of a bioassay for engineered nanoparticles (ENPs) in the environment

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ABSTRACT

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As the global production and use of the nanoparticles increases, it is certain that the engineered nanoparticles will be released into the environment. However, their effects on the humans, other organisms, and ecosystems are still unknown. In order to understand the potential toxicity and bioavailable concentration of the metal-based engineered nanoparticles or toxic trace metals in the environment, *Chlamydomonas rehardtii* was utilized as a study model to construct the bioassay. Although *C. reinhardtii* primarily serves as a model organism in biology research, the prevalent reporter genes such as GFP or luciferase are poorly expressed in this organism. No efficient bioluminescent or biofluorescent reporters are available for constructing our bioassay, thus, an endogenous gene, the *CRLpcr-1* gene which encodes protochlorophyllide oxidoreductase, as a reporter gene was first evaluated and expected to be served as a useful and robust reporter gene in *C. reinhardtii*. The 4.9 kb transgene including the nickel inducible promoter, the *Cyc6* promoter, the reporter gene, the *CRLpcr-1* gene, and the selectable marker was assembled using homologous recombination in *Saccharomyces cerevisiae*. The results showed that *C. reinhardtii* failed to express the *CRLpcr-1* transgene probably due to the transcriptional gene silencing mechanism. In future, a new strain that is defective in transcriptional silencing which is genetic crossed with a new mutant strain (*pc1, y7, cw15, arg7)* with flagella could be employed to construct our bioassay.

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List of Acronyms

AOT4	Amino acid transporter
ATP	Adenosine-triphosphate
bp	Base pair
Cd	Cadmium
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
hr	Hour
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria Broth
M	Molar
ml	Milliliter(s)
mM	Millimolar
NIT1	Nitrilase 1
OD	Optical Density
PCR	Polymerase chain reaction
RBCS2	Ribulose Bisphosphate Carboxylase/Oxygenase Small Subunit 2
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
rpm	Rotations per minute
TE	10 mM Tris, 1 mM EDTA
UV	Ultraviolet
µg	Microgram
µl	MicroLiter
µM	Micromolar

Introduction

1.1 Toxicity of nanoparticles

Nanoparticles (NPs) are small invisible particles whose sizes are smaller than 100nm. Due to their extremely small size, NPs have the ability to penetrate physiological barriers of the organisms and may result in the damage in them. Although, NPs are widely used in many consumer products such as cosmetics, drug carriers, clothing, medical device and biosensors (Wang, Zhu et al. 2012, Yazdi, Najafi et al. 2012), their health effects are still unknown. Some NPs have been previously considered safe, but revealed adverse health effects recently. For example, silver NPs which are widely used as an antibacterial agent are found to be toxic to human cells. Silver NPs have been found in the blood of patients who suffer blood diseases (Gatti, Montanari et al. 2004). Toxicity of metal-based NPs have been most concerned recently and widely studied in many organisms, yet the mechanisms of toxicity are still unclear. Currently, there are no efficient methods to quantify the concentration of NPs in the environment. And the questions about the toxic effects to the organisms caused either by NPs themselves (nanotoxicity) or by the dissolved metals still cannot be answered. Inversely, whether the aggregation effect of NPs could affect the toxicity is under exploited.

To understand the toxicity of NPs, previous study that used the complementary transcriptome profiling technique has revealed biomarker genes differentially expressed when exposed to the trace cadmium (Simon, Descombes et al. 2008). The transcriptome bioassay which examines the expression level of mRNAs in given cells can be used to as early warning indicators for the environmental effect and to identify molecular

mechanisms revealing toxicity. Moreover, the transcriptomic sequencing was also utilized to evaluate the toxicological effects of exposure to different metal-based NP species in *C. reinhardtii*. The study revealed that exposure to different metal-based NPs caused different toxicological effects on organisms. For example, nano-ZnO induced oxidative stress, whereas, nano-Ag damages the cell wall structure in *C. reinhardtii* (Simon, Domingos et al. 2013).

In our Study, we aim to develop a bioassay for detecting metal-based NPs in the environment using *C. reinhardtii* and to use this bioassay to understand the toxicity of NPs as well as their physiochemical and biological mechanisms.

1.2 *C. reinhardtii*

C. reinhardtii is an ideal organism for constructing our bioassay, since *C. reinhardtii* is unicellular green alga which is popularly employed as a study model for photosynthesis, chloroplast biogenesis, and flagella function. In addition, *C. reinhardtii* is genetically well characterized: the nuclear, chloroplast, and mitochondrial genomes have been completely sequenced (Merchant, Prochnik et al. 2007). And the manipulation toolkit of *C. reinhardtii,* such as the nuclear and chloroplast transformation techniques, has been well established (Kindle 1990). *C. reinhardtii* cells can survive in different environment from fresh water to soil where heavy metals pollutants exist. Moreover, they also have high robustness and tolerance to environmental conditions. Compared to the yeast or bacteria cells, they would not detoxify the medium (Harris, Stern et al. 2009).

1.3 A review of common reporter gene systems

The construction of our bioassay is based on the reporter gene system. Over the last two dedicates, reporter gene systems have greatly contributed to molecular and cellular research. A reporter gene is a gene which can be introduced into organisms and whose expression gives a response which is easy to quantify. Reporter gene systems are often utilized to study the *cis*-acting or *trans*-acting elements for gene transcription, pre-mRNA processing, and the translation (Alam and Cook 1990, Ignowski and Schaffer 2004). They also can be used to monitor transfection efficiencies, to identify protein-protein interaction, the protein subcellullar location, and expression patterns in specific tissues and developmental stages (De and Gambhir 2005).

Generally, in the reporter gene system, the reporter gene is fused to a promoter sequence or a gene of interest. The resulting chimera is constructed in a vector which is then transformed into the genome of the organism of interest. In such a tranformant, the expression of the reporter gene can be quantified by measuring the levels of reporter mRNA, protein product, enzymatic activities of the reporter protein, or by visualization of a light signal (as fluorescence or luminescence). Proteins that emit such a light signal are as green fluorescent protein (GFP) and luciferase signals (Rosenthal 1987).

1.3.1 Green fluorescent protein

GFP was discovered in the jellyfish, *Aequorea victoria* (Shimomura, Johnson et al. 1962) and has been widely engineered as a reporter protein for a variety of organisms from prokaryotes, such as bacteria to eukaryotes, including both plants and vertebrates (Matsuda, Yanagisawa et al. 2013, Xiong, Li et al. 2013, Zhou, Yang et al. 2013). Yellow

fluorescent protein (YFP), cyan fluorescent protein (CFP), and blue fluorescent protein (BFP) are also the three engineered derivatives from GFP generated by mutagenesis (Nagai, Ibata et al. 2002, Alvarez, Levin et al. 2010, Lee, Lim et al. 2013).

1.3.2 Applications of GFP

Being a most popular and useful biological tool, GFP can be utilized in diversely heterologous systems such as *Escherichia coli* and *Caenorhabditts elegans*, as it is well characterized, shown to be extremely thermostable, and have a broad pH tolerance range (Tsien 1998). The wild-type jellyfish GFP can emit strong and visible green fluorescence when excited with UV or blue light (Heim and Tsien 1996). The first application of GFP was as a reporter gene to detect gene expression *in vivo*, in *C. elegans*. It successfully confirmed the expression patterns of the *mec-7* promoter, which drives the expression of ß-tubulin in the mechanosensory neurons (Chalfie, Tu et al. 1994). GFP as a tag which is fused to domains or host proteins can be exploited to detect their subcellular localizations (Kato, Kawasaki et al. 2010, Chen, Shaffer et al. 2013). GFP has been successfully targeted to the proteins from almost every major organelle of the cell, such as the plasma membrane in insect cells (Chen, Shaffer et al. 2013), the endoplasmic reticulum, the Golgi apparatus in plant cells (Bubeck, Scheuring et al. 2008), the mitochondria in mammalian cells (Prowse, Chong et al. 2012), and the nucleus in yeast cells (Andoh, Oshiro et al. 2006). Moreover, codons of the GFP gene have been optimized to increase the gene expression level in mammalian and yeast systems (Zolotukhin, Potter et al. 1996, Cormack, Bertram et al. 1997).

GFP based imaging system is an extraordinarily powerful tool to explore cellular activities, such as chromosome assembly, segregation and cytokinesis (Verbrugghe and

Chan 2011). In addition, the phenomenon of fluorescence resonance energy transfer (FRET) between GFPs of different color has been exploited and extensively used in the study of protein-protein interactions (Malik, Ritt et al. 2013).

The advantage of the GFP reporter system is that the system is autofluorescence of the protein without any substrate requirement (Heim and Tsien 1996). However, GFP protein acts stoichiometrically, thus the reporter signal is not naturally amplified. Powerful fluorescent microscopes are required for detecting gene expression driven by low activity promoters (Schenborn and Groskreutz 1999).

1.3.3 Luciferases

The most commonly exploited luciferases come from the bacteria, the firefly, and the sea pansy (de Wet, Wood et al. 1985, Baldwin, Christopher et al. 1995). The bacterial luciferases are the heterodimeric proteins consisting of a 40 kDa α-subunit and a 37 kDa β-subunit, which are encoded by the *luxA* and *luxB* genes, respectively (Stewart and Williams 1992). As the bacterial luciferase cannot produce continuous emission light and are poorly expressed in mammalian cells, their applications as the reporter genes in luminescent assays are limited. However, the *Luc* gene from the firefly, *Photinus pyralis*, is one of the most frequently used reporter genes in biological laboratory due to amenability to manipulation. The enzymatic protein products of the *Luc* gene from firefly, a monomer of 60 kDa, can convert the chemical energy to visible light with a high efficiency. The accomplishment of the enzymatic light-producing reaction requires ATP, luciferin and oxygen as substrates (Green and McElroy 1956). The characteristic of the absolute requirement for ATP as a substrate has been used to develop bioluminescence assays for ATP detection (de Wet, Wood et al. 1985). Another member of luciferase

family, *Renilla* luciferase (Rluc), was isolated from the sea pansy, *Renilla reformis*. Compared to the firefly luciferase, the enzymatic reaction of *Renilla* luciferase uses a different substrate, coelenterazine, but does not require ATP to produce luminescence (Schenborn and Groskreutz 1999).

1.3.4 Applications of luciferases

Both the firefly luciferase and the sea pansy luciferase are remarkable indicators for gene expression, as they lack post-translational modification, exhibit fast enzymatic reaction. Thus, the luciferase reporter system is an excellent tool not only for detecting the low level gene expression, *in vivo* or *in vitro,* but also for detecting protein-protein interactions using two-hybrid screening. Luciferases are also mainly used to track the tumor cells, *in vivo,* in mammalians by live imaging system for monitoring tumor growth kinetics and treatment (Choy, Choyke et al. 2003, Doyle, Burns et al. 2004, De and Gambhir 2005).

Luciferase reporter genes are compatible to many organisms. Both the firefly luciferase and the sea pansy luciferase can be used in the promoter deletion analysis in mammalian cells for identifying important regulatory regions involved in transcriptional control of gene expression (Xu, Kanagaratham et al. 2013). In *C. elegans*, luciferases were contributed a lot to study of cell signaling pathway (Ludewig, Kober-Eisermann et al. 2004). Luciferase gene can also serve as reporter genes to study the regulatory elements in higher plants such as *Arabidopsis* and *Nicotiana* (Ono, Kusama et al. 2011).

The advantage of luciferases as reporter gene is that they have high sensitivity and have no heat liability (Yagi 2007). However, the light-producing enzymatic reaction of luciferase requires exogenous substrate and oxygen, and the luciferase signals must be

measured immediately while the reporter proteins are still active (Yagi 2007).

1.3.5 β-galactosidase (β-gal)

β-gal is a common reporter protein encoded by the *LacZ* gene from *E. coli*. β-gal is a tetrameric enzyme with subunit size of 116 kDa (Hall, Jacob et al. 1983). This enzyme can hydrolyze various β-galactosides sugars, and the enzymatic activities of β-gal can be assayed with different specialized substrates and quantified with a spectrophotometer, a fluorometer or a luminometer. One of the substrates, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), is hydrolyzed to produce the yellow-color precipitates and normally used in a colorimetric enzyme assay. Another substrate, 5-bromo-4-chloro-3-indolyl galactoside (X-gal), can be hydrolyzed by β-gal releasing a free indolyl, which is further oxidized into an indoxyl whose precipitates show blue color (Lim and Chae 1989, Juers, Matthews et al. 2012). Based on their chromogenic features, both ONPG and X-gal can be used to identify the transfection efficiency, the cell specificity, or the promoter expression level of cells producing β-gal (Schenborn and Groskreutz 1999).

1.3.6 Applications of β-gal

β-gal is commonly used as a reporter gene fused with a promoter of interest or a gene of interest to monitor gene expression under the specific condition or within a certain cell or organelle. Based on the α-complementation phenomenon of the *LacZ* gene, a powerful technology called blue-white screen were developed and are widely used to detect the recombinant bacteria in molecular cloning experiments (Juers, Matthews et al. 2012). In bacteria two-hybrid system, β-gal can also identify protein-protein interactions

(Schenborn and Groskreutz 1999).

Due to the potentially toxic chemicals for the assay and requirement to lyse eukaryotic cells in order to measure its activity or protein level, β-gal cannot be applied in real-time detection systems (Ghim, Lee et al. 2010).

1.4 Reporter genes in *C. reinhardtii*

Although the common reporter genes such as the GFP gene, luciferase gene, and *LacZ* gene can be successfully expressed in many organisms, they are poorly expressed in *C. reinhardtii*. This phenomenon has prevented the usage of the common reporter genes to study the gene expression or protein localization in *C. reinhardtii*. The possible reasons for the failure of expression of the foreign genes in *C. reinhardtii* are due to the epigenetic silencing of gene expression, such as methylation, the lack of adequate promoters, enhancers or introns required for the RNA processing, and inappropriate codon usage (Harris, Stern et al. 2009). For examples, the GFP gene which is a standard reporter gene expressed in other organisms but is poorly expressed in *C. reinhardtii* cells (Fuhrmann, Oertel et al. 1999). And the luciferase gene from *R. reniformis* which was adapted to the codon usage of *C. reinhardtii* was also synthesized, but limited studies have been published based on utilizing the codon-adapted luciferfase reporter (Fuhrmann, Hausherr et al. 2004).

1.4.1 Arylsulfatase

In *C. reinhardtii*, periplasmic arylsulfatases are produced in response to sulfur deprivation. Its activity can be detected by using 5-bromo-4-chloro-indolyl sulphate (X-SO4) as a chromogenic substrate (de Hostos, Schilling et al. 1989). The arylsulfatase

gene as a reporter gene was often utilized to study the *cis*-acting or *trans*-acting elements for gene transcription in *C. reinhardtii.* It was successfully expressed under the control of the β2-tubulin promoter (Davies, Weeks et al. 1992), and was then employed to explore the transcriptional regulation of the *tubB2* gene, which allowed mutated or removed sequences in the *tubB2* promoter region to be fused to the arylsulfatase gene. Several *tubB2 cis*-acting sequences in response to deflagellation were identified in these experiments (Davies and Grossman 1994). Arylsulfatases gene was also successfully expressed when fused to the *NIT1* promoter (Ohresser, Matagne et al. 1997). An insertionally tagged mutant library was created using the arylsulfatase gene as a reporter to identify the functional genomics genes regulating the nitrate assimilation pathway. Many putative genes were identified in this study indicating that a complex network of signaling proteins and derivatives of ammonium have the effects on the nitrate assimilation pathway (Gonzalez-Ballester, de Montaigu et al. 2005).

1.4.2 *aadA*

A bacterial gene, aminoglycoside 3"-adenyltransferase *(aadA)* conferring spectinomycin resistance from *E. coli,* is able to express in the chloroplast in *C. reinhardtii.* Being as a portable cassette, the *aadA* gene can be used to study the chloroplast reverse genetics to identify the unknown gene functions, because it is able to interrupt the genes whose function is needless under appropriate conditions (Goldschmidt-Clermont 1991). Being as a reporter gene, the *aadA* gene can be expressed under the control of the *RBCS2* promoter sequence. The chimeric construct can be stably integrated into the nuclear genome and showed the spectinomycin-resistance phenotype. But such phenotype was unstable; *aadA* transcription was inactivated when transformants

were not maintained on antibiotic condition (Cerutti, Johnson et al. 1997, Cerutti, Johnson et al. 1997).

1.4.3 *BLE*

The *BLE* gene isolated from *Streptoalloteichus hindustanus* confers resistance to antibiotics including tallysomysin, phleomycin, zeomycin, and bleomycin. It has become a dominant marker for both prokaryotes and eukaryotes. The heterologous *BLE* gene can stably integrate into the *Chlamydomonas* genome and express under the control of the endogenous promoter *RBCS2* (Stevens, Rochaix et al. 1996). In addition, the gene has been engineered to significantly increase the transformation efficiency and gene expression level by introducing the first intron of the *RBCS2* gene into the coding region of the *BLE* gene (Lumbreras, Stevens et al. 1998). Such modified chimeric marker has become a powerful molecular tool for *C. reinhardtii* studies.

1.5 Algae-based biosensor/bioassay

1.5.1 General background of biosensor/bioassay

A biosensor is kind of a device constructed based on the bioassay. Biosensors are analytical device widely employed in the medicine and food industry for the control of manufacturing processes. More recently, many biosensors/bioassays have been applied to detect and monitor the pollutants in the environment. It is generally composed of two parts: a bioreceptor, the biological sensing element which interact with the analytes, and a transducer transforming the signal from bioreceptor into another signal that is sensitive, easily measured and has minimal background noise (Yagi 2007). The bioreceptor are

mainly made of enzymes, antibodies, tissues and microorganisms (Ruan, Foo et al. 2008, Sassolas, Leca-Bouvier et al. 2008, Zhao, Lei et al. 2010). The major advantages of the ideal biosensors are their good sensitivity, specificity, and portability.

Detecting and monitoring systems which can detect the toxicants at very low concentration levels are more desired than ever, as increasing harmful toxic pollutants such as heavy metals and herbicides are being spread in natural ecosystems. Enzymatic biosensors are frequently utilized due to their high specificity; nevertheless, the enzyme purification process is laborious, time-consuming and expensive, and the enzymes are unstable in certain condition (Hanke, Wittmer et al. 2010, Stankovic and Jovic 2012). Compared to the enzyme-based biosensor, the cell-based biosensors are more ubiquitous and more stable in nature environment (Yagi 2007). Generally, whole-cell biosensors based either on enzyme inhibition or chlorophyll fluorescence can be used for constructing real-time analysis and online monitoring (Perron, Qiu et al. 2012).

1.5.2 Applications of algae-based biosensors

Unicellular green algae are frequently employed for constructing the monitoring systems due to their high sensitivity and their beginning position in the food chain, which would provide a good biological marker of ecosystem pollution. Such biosensors are often utilized to detect the pollutants such as herbicides, pesticides and heavy metals in river or agricultural runoffs (Durrieu, Tran-Minh et al. 2006). The conventional algae-biosensor which is non-genetic engineered is commonly classified into two types: micro-conductometric algal biosensor and optical algal biosensor (Durrieu, Tran-Minh et al. 2006). The micro-conductometric algal biosensor is made based on changes of the conductivity in membrane resulting from membrane enzyme activities which produce

ionic species. The resulting signals can be captured by conductometric amplifier (Trebbe, Niggemann et al. 2001). And in the optical algal biosensor, the biological signals can be measured resulting from chlorophyll fluorescence produced by photosynthesis, esterase activity or phosphatase activity (Ferro, Perullini et al. 2012). The algae-biosensors are so sensitive and efficient that they are able to perform their best to reach to micromolar detection limit of polluants within short time. A new algal-bioassay based on chlorophyll fluorescence has been developed recently. It was capable of successfully detecting that the microcystin resulting from the cyanobacteria bloom in the contaminated environment can affect photosynthetic efficiency from 0.01 μg/mL within 15 minutes (Perron, Qiu et al. 2012).

1.5.3 Our proposed algae-based biosensor

In our study, we aim to create a novel algal bioassay using recombinant DNA technology. Recombinant DNA technology has been widely applied in constructing bacterial biosensors who particularly respond to the presence of the toxic chemicals or heavy metals through the synthesis of a reporter protein, such as luciferase, β-galactosidas, or GFP (Yagi 2007). However, limited reporters can be employed in our bioassay due to the foreign gene silencing mechanisms. Thus, finding an available reporter gene which can be stably expressed in *C. reinhardtii* is a key point for our study. An endogenous *C. reinhardtii* gene, the *CRLpcr-1* gene which encodes protochlorophyllide oxidoreductase involving in chlorophyll biosynthesis pathway, was utilized as a reporter gene. The *CRLpcr-1* gene was fused to a metal-inducible or nanoparticle-inducible promoter which was then introduced into the yellow *C. reinhardtii* mutant strains that lack the essential enzymes to synthesize chlorophyll (Li and Timko

1996). After the mutants respond to the specific heavy metal or nanoparticle, the *CRLpcr-1* gene would be expected to be expressed and produce chlorophyll which results in the color change of the mutants from yellow to green.

Previous study in the collaborated project using global transcriptome profiling in *C. reinhardtii* identified genes that are induced in response to concentrations of environmentally relevant ionic form of Cd. A group of 10 genes expressed differentially in response to micromolar Cd^{2+} was shown to be specific to the Cd exposure and strongly correlated to its bioaccumulation (Simon, Descombes et al. 2008). Moreover, transcriptome profiling by mRNA deep sequencing was also employed to characterize specificity of these Cd *Chlamydomonas* genes when they are exposure to multiple metal solution including Ag nanoparticle or the quantum dot (Hutchins, Simon et al. 2010). Thus, the ultimate goal of our part in the collaborated project is to utilize such bioassay to quantify and detect Cd nanoparticle or Cd^{2+} in the contaminated environment and to propose an early-warning system before Cd causes irreversible damage in the environment. Therefore, the bioassay is expected to be sensitive to the environmentally relevant concentrations of the metals of interest, and also to be specific to the target metals which should not be influenced by other metals. In addition, the bioassay should be an easily manipulated, portable and inexpensive device.

1.5.3.1 The *Cyc6* promoter

Because it was the first time the *CRLpcr-1* gene was utilized in our study for constructing the bioassay, in order to validate the new reporter gene *C. reinhardtii*, a nickel-inducible promoter *Cyc6* was utilized to construct the chimera. The endogenous *Cyc6* gene encodes an electron transfer catalyst called cytochrome *c*6, which is a redox

carrier of the thylakoid lumen (Howe, Schlarb-Ridley et al. 2006). The expression of the *Cyc6* gene can be activated by copper depletion or by nickel addition (Quinn, Kropat et al. 2003). The *Cyc6* promoter has been used to design inducible promoter systems in *C. reinhardtii* cells, and the promoter was shown to be sufficient for nickel responsiveness with a heterologous gene in previous studies. For example, the *Cyc6* promoter has been successfully utilized to drive expression of the flagellar radial spoke complex gene (*RSP3*) to rescue the motility of *C. reinhardtii* paralyzed mutants (Ferrante, Diener et al. 2011). Thus, in our study, the *CRLpcr-1* gene was expected to be expressed in *C. reinhardtii* driven by the *Cyc6* promoter under the nickel induction, so that the *CRLpcr-1* gene reporter gene could be used for the bioassay detecting the metal-based NPs in the environment, when it is fused with different nanoparticle-inducible promoters. For example, the *CRLpcr-1* gene could be fused with the *AOT4* promoter to construct a specific, sensitive, simple bioassay for *in situ* measurement of relevant environmental Cd^{2+} concentration. The *AOT4* gene encoding a putative amino acid transporter and this gene was shown to have the highest transcriptional level of induction by Cd^{2+} among the 10 genes in the transcriptome profiling study (Simon, Descombes et al. 2008). In our previous study, *AOT4* promoter region was validated by northern blot, and was attempted to fuse with the reporter gene luciferase, but this fusion reporter system was failed to express measurable signal (Soowamber, Madhav 2012).

1.5.3.2 The *CRLpcr-1* gene and chlorophyll biosynthesis pathway

The chlorophyll biosynthesis pathway is a highly complex process. Protochlorophyllide (pchlide) is the immediate precursor to chlorophyll which is originated from glutamate. The conversion of pchlide to chlorophyll is a key step for

chlorophyll formation. This process is catalyzed by NADPH-protochlorophyllide oxidoreductase (POR) enzyme which encoded by the *CRLpcr-1* gene. POR is a thylakoid membrane-associated protein which consists of bound NADPH and protochlorophyllide *a* in a ternary complex (Murakami and Ikeuchi 1982). In the reduction process, protochlorophyllide *a* is converted to chlorophyllide *a* through light-dependent pathway or light-independent pathway (Choquet, Rahire et al. 1992, Suzuki and Bauer 1992). In flowering plants, the reduction process is a light-dependent reaction catalyzed by light-dependent protochlorophyllide reductase (LDPOR) (Murakami and Ikeuchi 1982). However, in green algae, there is another light-independent pathway which catalyzed by light-independent protochlorophyllide reductase (LIPOR) (Suzuki and Bauer 1992). And the cyanobacteria only contains the light-independent pathway producing chlorophyll (Suzuki and Bauer 1995).

C. reinhardtii depends on both light-independent and light-dependent pathway to produce chlorophyll. In the light-independent pathway, three genes, *chlB*, *chlL*, and *chlN,* encode the essential enzymes for chlorophyll production in the dark environment (Choquet, Rahire et al. 1992, Suzuki and Bauer 1992, Li, Goldschmidt-Clermont et al. 1993). Besides these three genes, additional seven genes called *Y* genes are needed for translation of the *chlL* gene. Mutation (*y7*) in any of the seven genes (*Y* genes) would block chlorophyll synthesis pathway. And the mutant cells would accumulate pchlide which grow in yellow color in the dark (y phenotype) (Li and Timko 1996). In our bioassay, *pcI* (mutation in *CRLpcr-1* gene), *y7* mutant cells contain mutations in both light-dependent and light-independent pathways. Without LDPOR enzymes, the mutant cells are sensitive to light (Li and Timko 1996). Expression of the wild-type *CRLpcr-1*

gene in the *pcI, y7* mutant strains are expected to restore the wild-type phenotype of *C. reinhardtii* strains.

1.5.3.3 DNA Assembler method

A new method called DNA Assembler technique was used to create our reporter construct containing the *CRLpcr-1* reporter gene, the metal inducible promoter *Cyc6*, and a selectable marker. In synthetic biology, the conventional methods used to combine the genetic elements frequently involve in multiple steps: PCR amplification or fusion PCR, cloning and transformation techniques. However, in some cases, these methods are time-consuming and inefficient, and methods relying on the special restriction enzymes would prevent recombination of large size DNA sequences. The DNA assembler method can rapidly synthesize large biochemical pathways by *in vivo* homologous recombination in *S. cerevisiae* cells (Shao, Zhao et al. 2009). It works in high efficiency through simple steps including DNA preparation and transformation. Shao (2009) used this technique successfully assemble three different biochemical pathways with the sizes of chimera ranging from 9 to 19 kb and with high efficiency (70%-100%) of the recombination on both plasmids and chromosomes.

1.6. Study purposes

1.6.1 Main project

We used *C. reinhardtii* and its endogenous *CRLpcr-1* gene as a reporter gene to develop a bioassay for metal-based nanoparticles (or toxic trace metals). We expected to use this bioassay to understand the nanoparticle toxicity and their physiochemical and

biological mechanisms. We also expected to use this bioassay to quantify the bioavailable or toxic NP species presenting in the environment.

1.6.2 Side project

In our side project, we used DNA assembler method to generate two constructs of *TBC1* genes including the *XRN1* and *PSBP9* genes fused with the selectable marker, respectively, to study and identify the function of *TBC1* gene. *TBC1* protein was known to interact with specific region of 5' untranslated region of chloroplast *psbC* mRNA to promote its translation and synthesize its protein product called CP43 subunit of photosystem II (PSII) complex (Rochaix, Kuchka et al. 1989). Previous studies have mapped and narrowed down the *TBC1* region to a 41 kb region including 5 sub-genes whose protein functions are still unknown (Stefanov 2006). Two genes, the *XRN1* and *PSBP9* (PsbP-like protein) gene, were considered as the best candidates for studying the function of the *TBC1* gene. In *Arabidopsis thaliana,* the PsbP protein was identified as an essential protein for PSII complex assembly or stability (Yi, Hargett et al. 2007). The *XRN1* protein was predicted as 5'-3' exonulease orthologue (Stefanov 2006).

Materials and Methods

2.1 *C. reinhardtii* strains and Culture conditions

C. reinhardtii mutant strains #1, #3, #6, #20, #27, #45 (*pc1, y7, cw15, arg7*), and *tbc-1* (*tbc1-F34*) were obtained from Dr. Zerges. The strains were cultured in tris-acetate-phophate (TAP) medium supplemented with 55 µg/ml argnine and 1% D-Sorbitol in the dark. Mutant strain, CC-3964 (*pcI, y7*) obtained from *Chlamydomonas* Resource Center (University of Minnesota, USA) was cultured in TAP, 1% D-Sorbitol medium in the dark. Wildtype strains, CC-620, CC-621, CC-503 provided by Dr. Zerges were maintained on 1.5% (w/v) TAP agar plates under the constant light.

2.2 *S. cerevisiae* strains, Plasmids and Culture conditions

S. cerevisiae competent cells were used as the host for DNA assembly. The pGREG506 plasmids were served as vectors after they were linearized by AscI and KpnI enzymes. Both cells and plasmids were kindly provided by Dr. Andy Ekins at Concordia University, Montreal. Plasmids were purified using Gene Jet plasmid miniprep kit (Fermentas) or Alkaline- lysis plasmid extraction method (Sambrook and Russel, 2001). *S. cerevisiae* cells were grown in YPD medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose.

2.3 DNA Assembler Methods

2.3.1 PCR

The promoter *Cyc6* and the coding region of the *CRLpcr-1* gene were amplified from genomic DNA isolated from *C. reinhardtii* CC-1618 strain; selectable markers, the *BLE* gene and cDNA of the *ARG7* gene, were amplified from pSP124S and pRb-ARGs plasmids by PCR, respectively. Different primer sets and PCR parameters are showed as below:

2.3.1.1 *Cyc6* PCR

The primer **set1** for the promoter *Cyc6* in the *Cyc6: CRLpcr-1-BLE* chimeric construct included the forward primer:

5'TAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGTTTAAACGGCGCG CCCTCGAGCAGAGGTTGGGAAT'3

and the reverse primer:

5'GGACTTGGCGGACATGGTGAGGGCCATGTCGATGGAGTAGGTTGAGT TAG'3

The primer **set2** for the promoter *Cyc6* in the *ARG-Cyc6: CRLpcr-1* chimeric construct included the forward primer:

5'TCTCGGTGGAGGGGTGGTCCTCTATCTCGAGCAGAGGTTGGGAAT'3

and the reverse primer:

5'GGACTTGGCGGACATGGTGAGGGCCATGTCGATGGAGTAGGTTGAGT TAG'3

The parameters for the *Cyc6* PCR were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 40 seconds and a final elongation at 72°C for 7 minutes.

2.3.1.2 *CRLpcr-1* PCR

The primer **set3** for the *CRLpcr-1* gene in the *Cyc6: CRLpcr-1-BLE* chimeric construct included the forward primer:

5'AGAACTAACTCAACCTACTCCATCGACATGGCCCTCACCATGTCC'3

and the reverse primer:

5'ATCCTGGTTTGGCTGCGCTCCTTCTTGAGGCTGCTATCCAATTATCC'3

The primer **set4** for the *CRLpcr-1* gene in the *ARG-Cyc6: CRLpcr-1* chimeric construct included the forward primer:

5' AGAACTAACTCAACCTACTCCATCGACATGGCCCTCACCATGTCC'3

and the reverse primer:

5'ATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTACCGCGGCC

GCTGAGGCTGCTATCCAATTATCC'3

The parameters for the *Cyc6* promoter were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute and 40 seconds and a final elongation at 72°C for 7 minutes.

2.3.1.3 *BLE* PCR

The primer **set5** for the *BLE* gene in the *Cyc6: CRLpcr-1-BLE* chimeric construct included the forward primer:

5'ACTGGATAATTGGATAGCAGCCTCAAGAAGGAGCGCAGCCAAAC'3

and the reverse primer:

5'ATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTACCGCGGCC GCTGGAGAAAGAGGCCAAAATC'3

The parameters for the *BLE* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 45 seconds and a final elongation at 72°C for 7 minutes.

2.3.1.4 cDNA of the *ARG7* PCR

The primer **set6** for cDNA of the *ARG7* gene in the *ARG-Cyc6: CRLpcr-1* chimeric construct included the forward primer:

5'TAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGTTTAAACGGCGCG CCCTCGCTCTCCAGCATCAAG'3

and the reverse primer:

5'AAGCGATTCCCAACCTCTGCTCGAGATAGAGGACCACCCCTCCAC'3

The parameters for cDNA of the *ARG7* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute and a final elongation at 72°C for 7 minutes.

The PCR reaction mixture (20 µl) consisted of 0.02 U/µl Phusion® DNA polymerase (Fermentas), 1 x Phusion® HF buffer, 200 µM dNTPs, 0.5 µM forward and reverse primer, 3% DMSO, 0.1–1 ng plasmid DNA or 500ng genomic DNA.

The PCR products were run on 0.8% agarose gels to confirm the quality of desired PCR products.

2.3.2 Gel Extraction

Expected PCR products and vectors were purified using Gene Jet Gel Extraction kit (Fermentas) according to the manufacturer's instruction and were mixed. To desalt and concentrate the DNA mixture, the DNA mixture was applied to Millipore's Amicon Ultra 10K centrifugal filter device (EMD Millipore). Manipulation was performed according to the manufacturer's instruction.

2.3.3 *S. cerevisiae* Transformation

A 25 ml of the YPD medium was inoculated with a 0.5 ml overnight *S. cerevisiae* culture and was shaken at 30°C and 200 rpm until OD_{600} reached to 0.75-1.0 by spectrophotometer. *S. cerevisiae* cells were harvested by centrifugation at room temperature at 2000 g for 1 minute. The supernatant was discarded and the cell pellet was washed with 25 ml of ice-cold sterile nanopure water, followed by another wash with 1ml cold 1 M sorbitol. The washing step was repeated three more times, and the final cell pellet was resuspended in 250 µl 1 M sorbitol. An aliquot of 50 µl of *S. cerevisiae* cells together with 20 µl concentrated DNA mixture from the gel extraction step was electroporated in a 2 mm cuvette at 1.5 kV, 25 uF, 200 HMS electroporation conditions. The transformed cells were immediately mixed with 1 ml YPD medium and shaken at

30°C for 1 hour. After that, cells were harvested by centrifugation at 2000 g for 1 minute, washed with room- temperature 1 M sorbitol for several times and finally plated on a YNB (0.67% (w/v%) YNB (Sigma), 2% glucose) plate for selecting the transformants. The YNB plates were incubated for 2-3 days until colonies appear. Colonies were picked and maintained on 100 µg/ml G418 plates for *S. cerevisiae* plasmid purification.

2.3.4 *S. cerevisiae* Plasmid Purification

A 10 ml YPD medium supplemented with 100 µg/ml G418 was inoculated with a single colony and was shaken at 30°C for overnight. Transformed cells were harvested by centrifuge; the cell pellets were resuspended in 200 µl resuspension buffer in Gene Jet plasmid miniprep kit (Fermentas), 100 µl zymolase solution (0.3% (w/v) zymolyase (MP Biochmicals), 1.2 M sorbitol, 50 mM EDTA, pH 8.0), and 5 µl ß-mercaptoethanol. The mixture was incubated at 37°C for 1 hour. The pretreated *S. cerevisiae* cells were used for plasmid rescue using GeneJet plasmid miniprep kit (Fermentas). Manipulation was performed according to the manufacturer's instruction. Eluted DNA plasmid was dialyzed against distilled deionized H_2O on a MF^{TM} membrane filter (EMD Millipore) for 20 minutes, and the whole volume was electroporated into *E. coli* competent cells.

2.3.5 *E. coli* Transformation

500 ml pre-warmed LB medium were inoculated with 50 ml of overnight *E. coli* DH5α culture and were shaken at 37°C. When the OD_{600} of the culture reached 0.35-0.4, the flasks were rapidly transferred to an ice-water bath for 15 to 30 minutes. *E. coli* cells were harvested by centrifugation at 1000 g for 15 minutes at 4°C. The supernatant was discarded and the cell pellets were resuspended in 500 ml of ice-cold sterile nanopure

water and harvested by centrifugation at 1000 g for 20 minutes at 4°C. Followed by another wash with 10 ml of ice-cold sterile nanopure water, cells were collected by centrifuge at 1000 g for 20 minutes at 4°C and were resuspended in 1 ml of ice-cold GYT (10% (v/v) glycerol, 0.125% (w/v) *S. cerevisiae* extract, 0.25% (w/v) tryptone) medium. An aliquot of 40 µl of *E. coli* cells together with *S. cerevisiae* plasmids was electroporated in a 2 mm cuvette at 2.5 kV, 25 uF, 200 HMS electroporation conditions. The transformed cells were immediately mixed with 1 ml LB medium supplemented with 100 µg/ml Ampicillin for recovery (Bio Basic Inc.) and were shaken at 37°C for 1 hour. The transformants were finally spread on a LB supplemented with 100 µg/ml Ampicillin plate for selection. Colonies were picked and cultured in 10 ml of LB 100 µg/ml Ampicillin medium for plasmid purification using Alkaline- lysis plasmid extraction method (Sambrook and Russel, 2001).

2.4 *C. reinhardtii* Transformation

2.4.1 Gamete Autolysin Preparation

C. reinhardtii strains CC-620, CC-621 (Chlamydomonas Resource Center) were cultured on TAP supplemented with 10% nitrogen plates for 24-48 hours under the direct light, respectively. Cells were collected and mixed in 8 ml TAP medium without nitrogen. The mixture was incubated under the light without shaking for 1-2 hour to allow the two gametes mating. The mating cells were examined under the microscope, and the cells were removed by centrifugation at 4000 rpm for 5 minutes. The supernatant containing autolysin was preserved in -80°C for digesting the cell walls of *C. reinhardtii* for transformation.

2.4.2 Electroporation Transformation

A 200 ml of TAP medium supplemented with 1% sobitol was inoculated with 20 ml of *C. reinhardtii* culture and was shaken at 24°C in the dark. Cells were harvested by centrifugation at 4000 rpm for 10 minutes until the cell density reached 1-2 $\times 10^6$ cells/ml. The cell pellets were resuspended in 1/25 volume of the autolysin medium supplemented with 60 mM sucrose and were shaken for 40 minutes. Cells were collected by centrifugation at 4000 rpm for 5 minutes and were resuspended in 2 ml TAP medium. An aliquot of 250 µl of *C. reinhardtii* cells together with 0.1-1 µg of DNA was electroporated in a 4 mm cuvette at 0.8 KV, 25 uF, with the shunt resistor off. The cuvettes were incubated on ice for 15 minutes prior to transformation. The transformed cells were then mixed with 15 ml TAP medium and incubated for 16 hours. Finally, cells were collected by centrifugation and plated on the selective plates.

2.4.3 Glass-bead Transformation

For glass-bead transformation, the procedures of transformation were performed the same as described in electroporation transformation method, except for the procedures that an aliquot of 300 µl of *C. reinhardtii* cells together with 0.1-1 µg of DNA was mixed with 300 mg glass-beads (Fisher) and were vortexed for 15 seconds for transformation.

2.5 Colony PCR

A single colony of transformed cells was picked, resuspended in 50 µl TE (10 mM Tris, 1 mM EDTA, pH 8) buffer, and was vortexed for 10 seconds. Cells were

incubated at 98°C for 10 minutes prior to incubation on ice for 1 minute. Cell debris was removed by centrifugation at 14000 rpm for 1 minute. 5 µl of the supernatant as a template was used for PCR reactions.

The primer set for colony PCR included the forward primer:

5'ACTGGATAATTGGATAGCAGCCTCAAGAAGGAGCGCAGCCAAAC'3

and the reverse primer:

5'ATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTACCGCGGCC GCTGGAGAAAGAGGCCAAAATC'3

The parameters for colony PCR were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 45 seconds and a final elongation at 72°C for 7 minutes.

2.6 Nickel Induction

Transformed cells were grown in 15 ml or 100 ml of the TAP medium and were induced at 3×10^6 cells/ml by adding 25 µM, 50 µM, or 75 µM of $NiSO_4$ for 48 hours. Samples for chlorophyll measurement were collected at a given time.

2.7 Chlorophyll Quantification

20-40 µl of Ni-inducible transformed cells were taken for measuring chlorophyll concentration. Chlorophyll was extracted in the 1 ml absolute methanol and each chl was measured using excitation light at 652 nm (Chlorophyll a) and 665 nm (Chlorophyll b) of a spectrophotrometer. The measuring values were into the following equation for calculating the chlorophyll concentration.

Chlorophyll (a+b) (μg/ml) = 22.12 xA_{652} + 2.71xA_{665} (Porra 2002)

2.8 DNA sequencing

The 4.9 kb *Cyc6-CRLpcr-1-BLE* chimeric construct was confirmed and sequenced by Innovation Center (McGill University and Genome Quebec); reverse and forward primes for sequencing are showed as follow (Table 1):

Sequences were analyzed by CodonCode Aligner software.

Forward Primers	Reverse Primers
5'CTCGAGCAGAGGTTGGGAAT'3	5'TGAGGCTGCTATCCAATTATCC'3
5'TCCAGGGTGTTACCACTTCC'3	5'CTGTAATCAAACACGCTGCAA'3
5'CCCTCTCTGGCTGACAAGTT'3	5'GACACGTAGCCCTTGGTGAT'3
5'GGCTTGCAAAAAGGTGGTTA'3	5'GTCCAGGTGCAGGATTGAGT'3
5'CCAAGCTGTGGGACATCTCT'3	5'GTCGATGGAGTAGGTTGAGTTAG'3
5'AGCTTGTGGCTTGGGAACTA'3	5'CCTGCTACATTGGCAACAGA'3

Table.1 Reverse and forward primers of *Cyc6-CRLpcr-1-BLE* construct for DNA sequencing.

2.9 RNA extraction

Cells treated by $NiSO_4$ for 24 hours were collected by centrifugation at 5000 rpm for 5 minutes. Cell pellets were resuspended in 1 ml TE (10 mM Tris, 1 mM EDTA, pH 8) buffer. Followed by another centrifugation at 5000 rpm for 2 minutes, cell pellets were resuspended in 0.5-0.6 ml of Tri-Reagent (Sigma). 70-80 mg sterile glass beads were added and vortexed for 15 seconds. Cells were then incubated at room temperature for 5

minutes and collected at 14000 rpm for 5 minutes. Followed by adding 0.1 ml of chloroform, aqueous phase was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then extracted twice with chloroform-isoamyl alcohol (24:1) solution. An equal volume of isopropanol was added and maintained at room temperature for 10 minutes. RNA was harvested at 14000 rpm for 10 minutes. RNA pellets were washed with 75% ethanol and then were resuspended in 20 µl of DEPC-treated HPLC grade H_2O. RNA was measured by spectrophotometer at 260 nm. And the RNA concentration was calculated using the following equation:

RNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (40 µg RNA/ml) (Gallagher 2001).

2.10 RT-PCR

RT-PCR was performed using SuperScript^{TM} III Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. The forward and reverse primer sets for RT-PCR are as below:

Set 1: forward primer: 5'ATGGCCCTCACCATGTCC'3, and the reverse primer: 5'CAGACGACCAGGGGAGTG '3

Set 2: forward primer: 5'AGTTTACAGCGCAAGGGAAC'3, and the reverse primer: 5'GTCGATGGAGTAGGTTGAGTTAG'3

2.11 DNA Assembler methods for TBC-1 project

2.11.1 PCR for the *XRN1-BLE* Construct

In order to prepare the *XRN1-BLE* fusion construct, coding region of the *XRN1* gene and the *BLE* gene were amplified from *C. reinhardtii* genomic DNA and the pSP124S plasmid using PCR, respectively. Due to the large size of the *XRN1* gene which is around 10 kb, the *XRN1* gene was divided for 6 fragments with overlaps. Different primer sets and PCR parameters are showed as follow:

The primer **set7** for the first fragment of the *XRN1* gene included the forward primer:

5'TAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGTTTAAACGGCGCG CCGCTTTATCCAGCCAAGTGGT'3

and the reverse primer:

5'AGCTTGATGCAGCCAAACTC '3

The parameters for the first fragment of the *XRN1* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute and 30 seconds and a final elongation at 72°C for 7 minutes.

The primer **set8** for the second fragment of the *XRN1* gene included the forward primer:

5'CTTACACACAGGCTGCTTCG'3

and the reverse primer:

5'CTAGCTTTTGATGGCGAAGG'3

The parameters for the second fragment of the *XRN1* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 52 °C for 30 seconds and extension at 72°C for 1 minute and 30 seconds and a final elongation at 72°C for 7 minutes.

The primer **set9** for the third fragment of the *XRN1* gene included the forward primer:

5'GCTGCCCTTGTATACCTTTGC'3

and the reverse primer:

5' CAGGGAGGATCAGAAACTGC '3

The parameters for the third fragment of *XRN1* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute and a final elongation at 72°C for 7 minutes.

The primer **set10** for the fourth fragment of the *XRN1* gene included the forward primer:

5'GCAGCGACTGCCAAACAC'3

and the reverse primer:

5'TTGGGCACCAGCTTCTGA'3

The parameters for the fourth fragment of the *XRN1* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute and a final elongation at 72°C for 7 minutes.

The primer **set11** for the fifth fragment of the *XRN1* gene included the forward primer:

5' GCAGTGGCTGGTGAAAAGC '3

and the reverse primer:

5' GTGGCGAATCCAGCAACTAT'3

The parameters for the fifth fragment of the *XRN1* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute and a final elongation at 72°C for 7 minutes.

The primer **set12** for the sixth fragment of the *XRN1* gene included the forward primer:

5'ACCTGCTGAAGGAGCTGAAG'3

and the reverse primer:

5' ATCCTGGTTTGGCTGCGCTCCTTCTCATCGTGCGGATTTTGTAGA'3

The parameters for the sixth fragment of the *XRN1* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 45 seconds and a final elongation at 72°C for 7 minutes.

The primer **set13** for the *BLE* gene in the *XRN1-BLE* construct included the forward primer:

5'GCCCATCTACAAAATCCGCACGATGAGAAGGAGCGCAGCCAAAC'3

and the reverse primer:

5'ATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTACCGCGGCC GCTGGAGAAAGAGGCCAAAATC'3

The parameters for the *BLE* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 45 seconds and a final elongation at 72°C for 7 minutes.

2.11.2 PCR for the *PSBP9-BLE* Construct

Chimera was generated by fusing the *PSBP9* gene and the *BLE* selectable marker, which were amplified from *C. reinhardtii* genomic DNA pSP124S plasmid using PCR, respectively. Different primer sets and PCR *PSBP9-BLE* parameters are shown as follow:

The primer **set14** for the *PSBP9* gene included the forward primer:

5'TAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGTTTAAACGGCGCG CCGTTGGCCGAGGCGTATAAT'3

and the reverse primer:

5' ATCCTGGTTTGGCTGCGCTCCTTCTGAAGGAGACGAAGGTGTTGC'3

The parameters for the *PSBP9* gene were as follow: an initial denaturation step at 98°C for 30 seconds followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 2 minutes and a final elongation at 72°C for 7 minutes.

The primer **set15** for the *BLE* gene in the *XRN1-BLE* construct included the forward primer:

5' TCGCTGCAACACCTTCGTCTCCTTCAGAAGGAGCGCAGCCAAAC'3 (94)

and the reverse primer:

5'ATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTACCGCGGCC GCTGGAGAAAGAGGCCAAAATC'3

The parameters for the *BLE* gene in the *PSBP9-BLE* construct were described above the same as the parameters for the *BLE* PCR in the *XRN1-BLE* construct.

The PCR reaction mixture (20 µl) consisted of 0.02 U/µl Phusion® DNA polymerase (Fermentas), 1 x Phusion® HF buffer, 200 µM dNTPs, 0.5 µM forward and reverse primer, 3% DMSO, 0.1–1 ng plasmid DNA or 500 ng genomic DNA.

The PCR products were run on 0.8% agarose gels to confirm the quality of desired PCR products.

Results

3.1 DNA assembler

Towards the development of bioassays for detecting nanoparticles in the environment, two types of chimeric genes were constructed based on the selectable makers utilized. The *ARG7* gene encodes argininosuccinate lyase, an enzyme in the biosynthesis of arginine. This gene or cDNA of its mRNA is able to complement the mutant deficient of *arg7-8* mutant strains of *C. reinhardtii* (Purton and Rochaix 1994, Auchincloss, Loroch et al. 1999). The selectable marker *BLE* encodes a protein conferring resistance to the antibiotics bleomycin and zeocin in the selective medium (Stevens, Rochaix et al. 1996). Each construct is comprised of the coding region of the *CRLpcr-1* gene which was placed under the control of the nickel-inducible *Cyc6* promoter and a selectable marker, either the *BLE* gene or cDNA of the *ARG7* gene. The schemes of the constructs are represented in Figure 1A for the *Cyc6: CRLpcr-1-BLE* fusion construct and Figure 1B for the *ARG-Cyc6: CRLpcr-1* fusion construct.

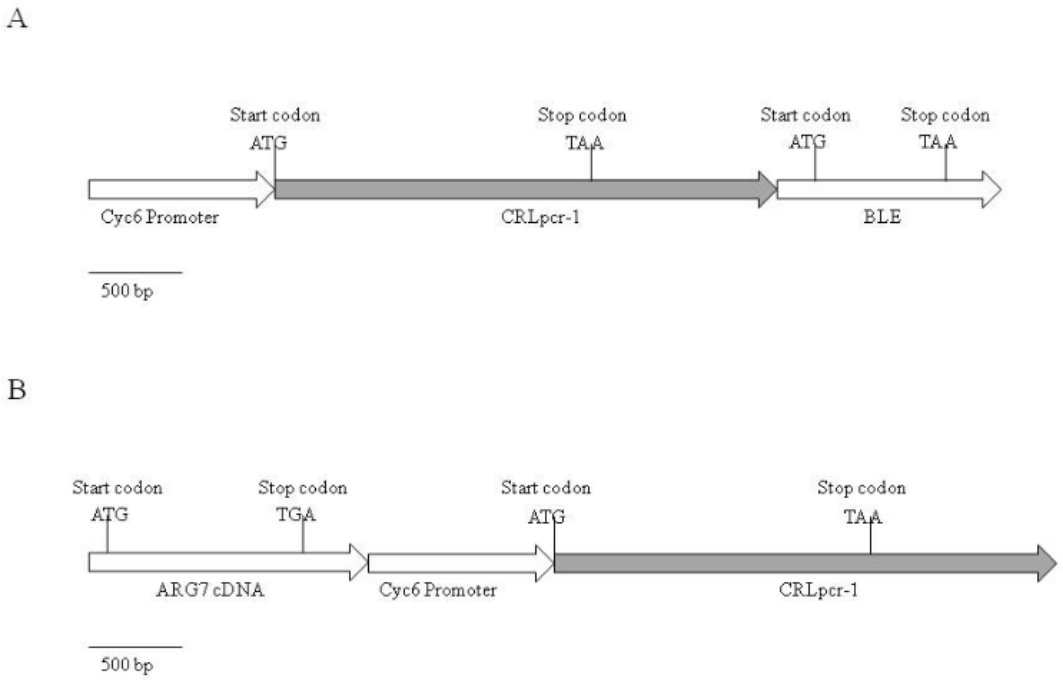

Figure 1. Constructions of (A) the *Cyc6: CRLpcr-1-BLE* and (B) the *ARG-Cyc6: CRLpcr-1* fusion genes.

3.1.1 DNA assembly for the *Cyc6: CRLpcr-1-BLE* construct

Constructs were made with a method called "DNA assembler" which uses the high frequency of homologous recombination in the *S. cerevisiae* to fuse transformed PCR fragments and a special plasmid vector in an order determined by homologous regions introduced at their end as described in detail below (Shao, Zhao et al. 2009). To fuse the *Cyc6* promoter to the structural part of the *CRLpcr-1* gene, I amplified the following fragments with PCR. The 5' end of the *Cyc6* promoter was designed to overlap with the vector backbone, while the 3' end was designed to overlap with the second *CRLpcr-1* PCR fragment at the 5' end. The *CRLpcr-1* PCR fragment was designed to overlap with the two flanking ones, and 3' end of the last *BLE* PCR fragment was

designed to overlap with the other end of the backbone. The size of the overlapping regions is 50 bp as required for efficient *in vivo* homologous recombination in *S. cerevisiae* cells. As shown in Figure 2, the PCR products of the expected sizes were obtained for the *Cyc6* promoter (1,008 bp), *CRLpcr-1* (2,769 bp), and *BLE* (1,229 bp).

Plasmid pGREG was digested with the restriction enzymes AscI and KpnI. The expected fragments were obtained: one smaller band of 1,500 bp and a vector backbone at around 6,000 bp (Lane V of Figure 2). To remove the non-specific amplicons and the primers, the upper 6000-bp band of the backbone together with the other PCR products was gel purified, concentrated, and desalted. The final PCR products were co-transformed into *S. cerevisiae* competent cells by electroporation. Transformation required that a functional plasmid to be generated within each transformant allowed the different genes assembled orderly into the backbone by homologous recombination.

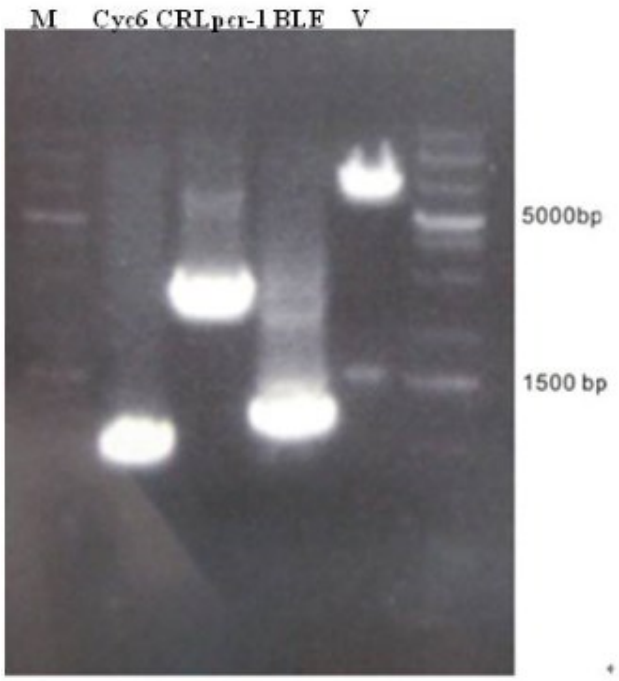

Figure 2. Agarose gel electrophoresis analyses of the PCR fragments containing the *Cyc6* promoter, the coding region of the *CRLpcr-1* gene and the *BLE* gene. M presents 1 kb plus marker (Fermentas); V presents the double digested vector.

A total of 14 colonies appeared on the selective plates 2-3 days later after the electroporation transformation. The colonies were randomly picked for the *S. cerevisiae* plasmid purification. The *S. cerevisiae* plasmids carrying the *Cyc6: CRLpcr-1-BLE* fusion construct were obtained from total DNA preparations by plasmid rescue in *E. coli*.

In order to confirm the correct assembly of the *Cyc6: CRLpcr-1-BLE* fusion construct in the plasmid obtained from *E. coli*, a set of PCR reactions was performed. As shown in Figure 3A, three pairs of primers annealing to the corresponding regions flanking each cassette were used as verification primers in diagnostic PCR. PCR reactions generating all three expected PCR (B1-B3) fragments with the correct sizes (as

shown in Figure 3B) indicate that the correct *Cyc6: CRLpcr-1-BLE* fusion construct was produced using the DNA assembler method.

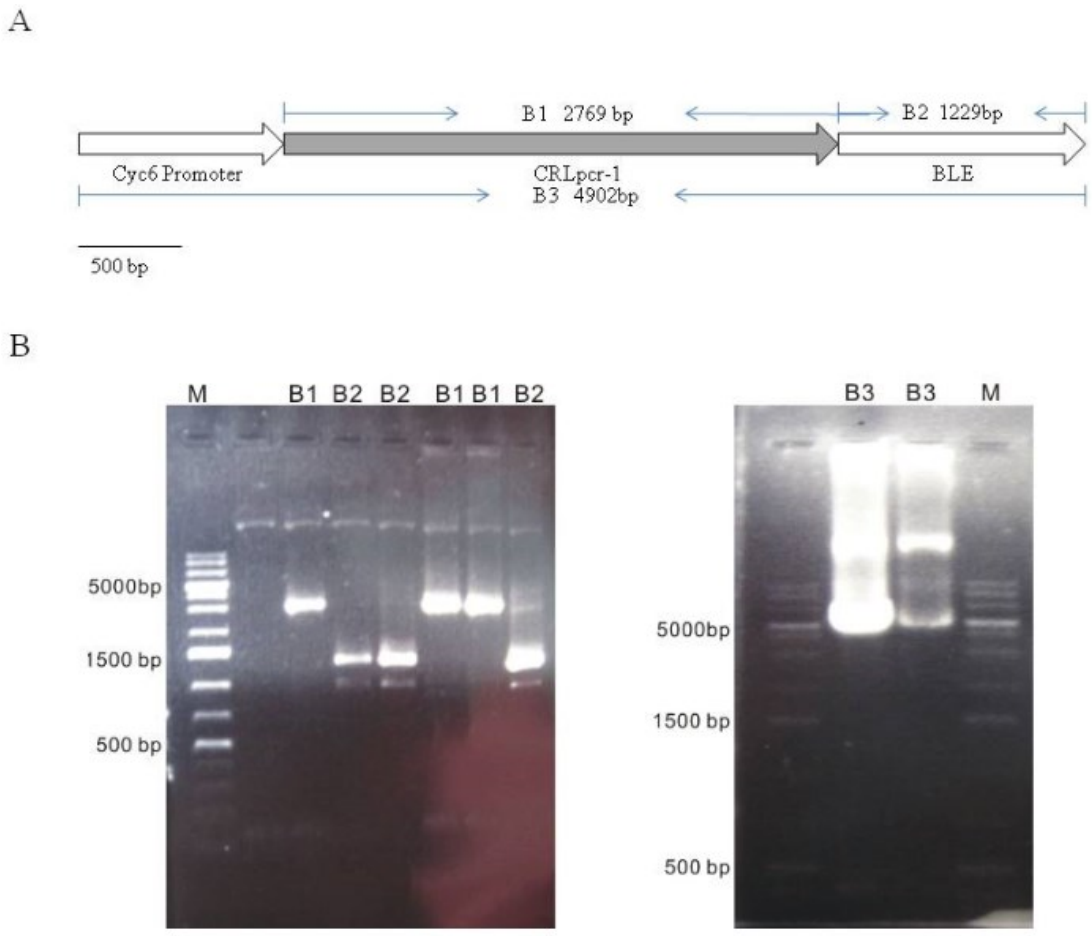

Figure 3. Confirmation of the correct assembly of the *Cyc6: CRLpcr-1-BLE* fusion construct. (A) The scheme of the *Cyc6: CRLpcr-1-BLE* fusion construct with expected sizes of the PCR fragments for confirming the correct DNA assembly. (B) Agarose gel electrophoresis analyses of the PCR fragments containing B1, the coding region of the *CRLpcr-1* gene (2,769 bp), B2, the *BLE* gene (1,229 bp), and B3, the whole fusion construct (4,902 bp). M indicates 1 kb plus marker (Fermentas).

3.1.2 DNA assembly for the *ARG-Cyc6: CRLpcr-1* construct

In the other *ARG-Cyc6: CRLpcr-1* fusion construct, the cDNA of the *ARG 7* gene was used as a selectable marker instead of the *BLE* gene. To create the fusion construct, the cDNA of *ARG 7*, the *Cyc6* promoter, and the coding region of the *CRLpcr-1* gene were also amplified by PCR. As shown in Figure 1B, the 5' end of the cDNA of the *ARG 7* gene was designed to overlap with the backbone of the vector, while the 3' end was designed to overlap with the *Cyc6* promoter sequence. The *Cyc6* promoter was designed to overlap with the two flanking genes, and the 3' end of the *BLE* gene was designed to overlap with the other end of the backbone of the vector. The expected PCR products of the cDNA of the *ARG7* gene (1,672 bp), the *Cyc6* promoter (983 bp), and the *CRLpcr-1* fragment (2,794 bp) were obtained as shown in Figure 4. The upper 6,000-bp band (Lane V of Figure 4) was from double digested vector and was gel-purified together with the other PCR fragments. These fragments and the linearized vector were then transformed into *S. cerevisiae* by electroporation for DNA Assembly.

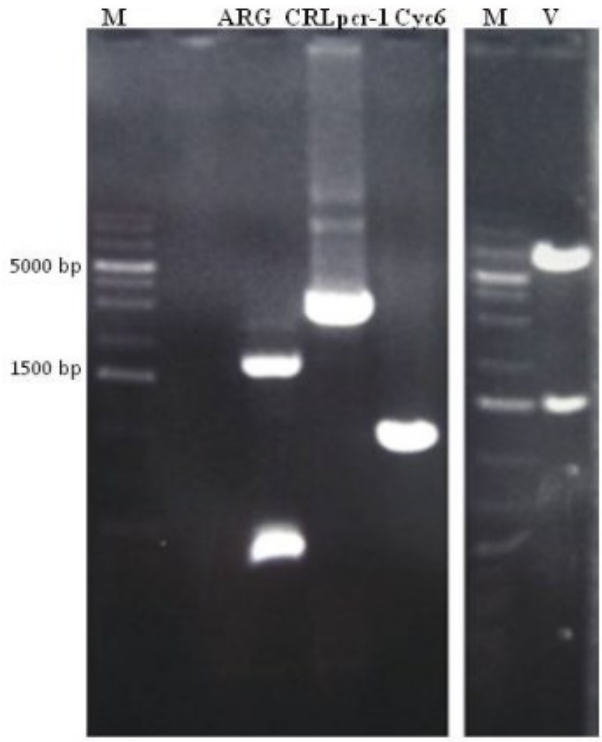

Figure 4. Agarose gel electrophoresis analyses of the PCR fragments containing the cDNA of the ARG 7 gene, the *Cyc6* promoter, and the coding region of the *CRLpcr-1* gene. M presents 1 kb plus marker (Fermentas). V presents the double digested vector.

A total of five colonies were obtained from *S. cerevisiae* transformation. The *S. cerevisiae* plasmids carrying the *ARG-Cyc6: CRLpcr-1* fusion construct were obtained by preparing total DNA in which was then used to transform *E. coli.* in order to rescue the plasmid and prepare sufficient quantities for the subsequent steps.

In order to confirm the correct assembly of the *ARG-Cyc6: CRLpcr-1* fusion construct in the plasmid obtained from *E. coli*, a set of PCR reactions was performed as shown in Figure 5A. PCR reactions generating all four expected PCR fragments (A1-A4) with the correct sizes (as shown in Figure 5B) indicate that the correct *ARG-Cyc6: CRLpcr-1* fusion construct was created using the DNA assembler method.

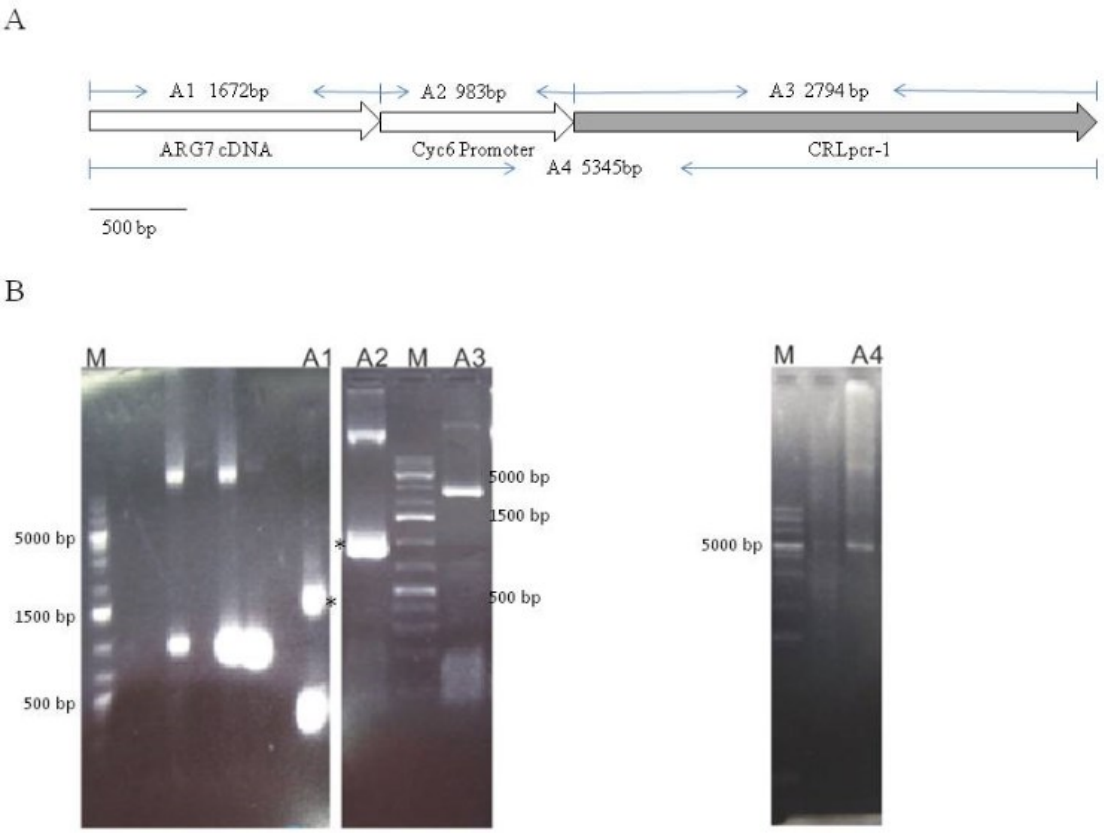

Figure 5. Confirmation of the correct assembly of the *ARG-Cyc6: CRLpcr-1* fusion construct. (A) The scheme of the *ARG-Cyc6: CRLpcr-1* fusion construct with expected sizes of the PCR fragments for confirming the correct DNA assembly. (B) Agarose gel electrophoresis analyses of the PCR fragments containing A1, cDNA of *ARG 7* (1,672 bp), A2, the Cyc6 promoter sequence (983 bp), A3, the coding region of the *CRLpcr-1* gene (2,769 bp), A4, the whole fusion construct (5,345 bp). M presents 1 kb plus marker (Fermentas). Symbol"*" indicates the specific PCR fragment.

3.2 *C. reinhardtii* Transformation

Either the plasmids carrying the *ARG7-Cyc6: CRLpcr-1* and the *Cyc6: CRLpcr-1-BLE* constructs, or the vector-removed gel-purified *ARG7-Cyc6: CRLpcr-1* and *Cyc6: CRLpcr-1-BLE* fragments were separately transformed to six *C. reinhardtii* mutant strains (#1, #3, #6, #20, #27, and #45). These strains (*y7, pcI, arg7, cw15*) carry mutations in the *CRLpcr-1* gene (*pcI*) and the gene encoding the light-independent POR (*y7*), so that regulated expression of the *CRLpcr-1*gene would be detected (Li and Timko 1996). They carry the *cw15* mutation which results in a cell wall defect which is required for transformation using the glass bead method (Kindle 1990). Finally, they carry the *arg7* mutation in the gene encoding argininosuccinate lyase, which results in auxotrophy for arginine (Debuchy, Purton et al. 1989). This mutation allows the use of the *ARG7* cDNA as a selectable marker for transformation (Auchincloss, Loroch et al. 1999). Transformation was performed using both glass-bead and electroporation methods, as different mutant strains have their preference for special transformation method. For example, transformation of strain #6 worked using the glass-bead method, while #20 was only transformable using the electroporation method (Table 2).

In order to evaluate the proper concentration of the antibiotic zeocin in the media for transformation selection, the pSP124S plasmids were transformed into the CC-1618 (*cw15, arg7*) strain using the glass-bead transformation method. The pSP124S plasmid carries the coding region of the *BLE* gene, which is under the control of the highly expressed endogenous *RBCS2* promoter in *C. reinhardtii* (Lumbreras, Stevens et al. 1998) The advantages of using the antibiotic *BLE* gene as a selectable marker are that (1) the size of the gene is small for cloning, (2) and it can express in *C. reinhardtii* and present

without the requirement of the selective pressure for the antibiotic for the *BLE* protein synthesis to occur (Lumbreras, Stevens et al. 1998). Different concentrations of antibiotic zeocin TAP medium, 1 μg/ml, 3 μg/ml, 5 μg/ml, 10 μg/ml, 15 μg/ml and 20 μg/ml, were prepared. Transformants having the pSP124S plasmids and the ones without the pSP124S plasmids were separately selected on these plates. Around 20 to 30 transformants carrying the pSP124S plasmids grew on the 10 μg/ml, 15 μg/ml and 20 μg/ml zeocin plates, while negative-control cells did not. Thus, the concentrations of 10 μg/ml and 20 μg/ml zeocin medium were used for transformation selection. Table 2 shows the summary results of the transformation of the *Cyc6-CRLpcr-1-BLE* plasmids into 6 different mutant strains. For three of them (#3, #6 and #20), the transformation worked. However, the transformation efficiencies for these strains were quite low; only one or two colonies were obtained for each-time transformation. Strains #27 and #45 grew poorly and showed low viability even in the dark. Therefore, this does not reflect the known light sensitivity of mutants lacking POR. Moreover, the 4.9 kb gel-purified construct was also transformed. It seems like that linearization of the plasmid did not significantly increase the transformation efficiency as the one obtained from the supercoiled plasmids.

In order to test the transformation efficiency of the mutant strains and the operating steps of the transformation, *Arg7.8* plasmids were transformed into both mutant strain CC-1618 (*cw15, arg7*) as the positive control and the quadruple mutant strain #6 (*pcI, y7, cw15, arg7*) using the glass-bead transformation method. CC-1618 is a strain easily transformable with *Arg7.8* plasmid, and it is often presented high transformation frequency. The results showed that more than 100 colonies from CC-1618 strain were obtained on the selective plates, and only 5 to 15 colonies were obtained from the #6

strain. This result revealed that the quadruple mutant strains have drastically lower transformation efficiency than the standard strain used for transformation. This was one of the major problems encountered in this project.

Genotype	**Electroporation Total No. of transformants**	**Glassbeads Total No. of transformants**	**Color change to green**
y7:pcI:arg:cw15 #1	0	0	-
y7:pcI:arg:cw15 #3	-	1	No
y7:pcI:arg:cw15 #6	-	6	No
y7:pcI:arg:cw15 #20	5	-	No
y7:pcI:arg:cw15 #27	-	0	-
y7:pcI:arg:cw15 #45	-	0	-
y7:pcI (CC-3964)	0	0	-

Table 2. Summary results of the transformation of 7 mutant strains with *Cyc6: CRLpcr-1-BLE* construct and the results of the color change of the cultures for nickel induction. Symbol "-" indicates not applicable since there were no transformants obtained from the transformation.

On the other hand, the co-transformation of *ARG7.8* plasmid with the non-selectable gene is a classical transformation method, but it is time-consuming, because it involves in many steps to screen for the desired colonies. Thus, in this study the *ARG7* cDNA was used for simplifying the transformation and selection procedures. A linear cDNA of *ARG7* lacking promoter sequences was shown to be capable to complement the *arg7* mutant in previous study (Auchincloss, Loroch et al. 1999), which indicates that it can be used in promoter-trapping experiments for our construct. After the transformation, unfortunately, I did not get any transformants even though this step was repeated and optimized at least ten times.

3.3 Confirmation of *C. reinhardtii* Transformation

The desired transformants with the *Cyc6: CRLpcr-1-BLE* construct were selected by the zeocin plates and screened using colony PCR; colonies were carefully picked and maintained. As the *Cyc6* promoter and the *CRLpcr-1* gene are endogenous sequences in *C. reinhardtii* cells, while the *BLE* gene is a foreign gene, it is much easier to tell if the fusion construct integrated in the transformed cells using the primers flanked at the both the end of the *BLE* gene for colony PCR. As shown in Figure 6, PCR reactions with colony cells yielded the expected 1,229 bp PCR fragments (Lane 1-3) having the same size as the ones (Lane 4-5) amplified from the pSP124S plasmid bearing the *BLE* gene. Negative control PCR reactions (without the cells) did not yield any PCR fragment (Lane 6). A total of eleven colonies were picked up and were performed colony PCR. Eight yielded the expected PCR products. Thus, the *C. reinhardtti* transformation successfully introduced the *Cyc6: CRLpcr-1-BLE* fusion construct in to the target strains.

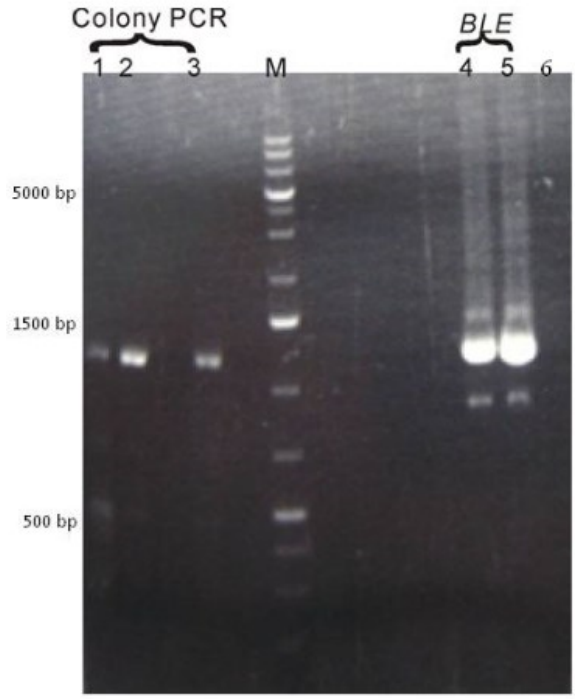

Figure 6. Agarose gel electrophoresis analyses of the colony PCR fragments of the *BLE* gene amplified from the transformants carrying the *Cyc6-CRLpcr-1-BLE* fusion construct (Lane 1-3), the PCR fragment amplified from pSP124Splasmid (Lane 4-5), and negative control from the transformants without the *Cyc6-CRLpcr-1-BLE* fusion construct (Lane 6)

3.4 Nickel Induction

The *Cyc6* promoter, a nickel-inducible promoter, was used for the design of the inducible promoter systems to evaluate the reporter gene *CRLpcr-1* in the bioassay. The *Cyc6* gene encoding an electron transfer catalyst can be transcriptionally activated by nickel addition or copper depletion (Quinn, Kropat et al. 2003). Inducible expression of the *Cyc6* promoter fused with the other endogenous gene has been reported before, but no data are available to our knowledge on the capacity of the *Cyc6* promoter to drive wild-type *CRLpcr-1* gene to complement *C. reinhardtii* (*pc1, y7)* mutants. The *Cyc6: CRLpcr-1-BLE* transformants were inoculated in TAP+R medium allowing optimal expression of the *Cyc6* promoter in the dark and induced at 3×10^6 cells/ml by adding 25 μM of $NiSO_4$ for 24 hours. We expected to see the color change of the culture turning from yellow to green when chlorophyll was produced from wild-type *CRLpcr-1* in *pc-1, y7 mutant* strains. But unfortunately, none of cultures of the *Cyc6: CRLpcr-1-BLE* transformants turned their color from yellow to green under the Ni induction as shown in Figure 7. To exclude the reason that the minimal amount of the nickel dissolved in the culture is not capable to express the *Cyc6* promoter, higher concentrations of $NiSO_4$ (50 μM, 75 μM) and longer induction time (48 hours) were applied for the Nickel induction. As time went on, the color of the cultures became darker, because the cell density increased, but no color change occurred. This results indicate that the chlorophyll might not be produced in the *Cyc6: CRLpcr-1-BLE* transformants during the nickel induction.

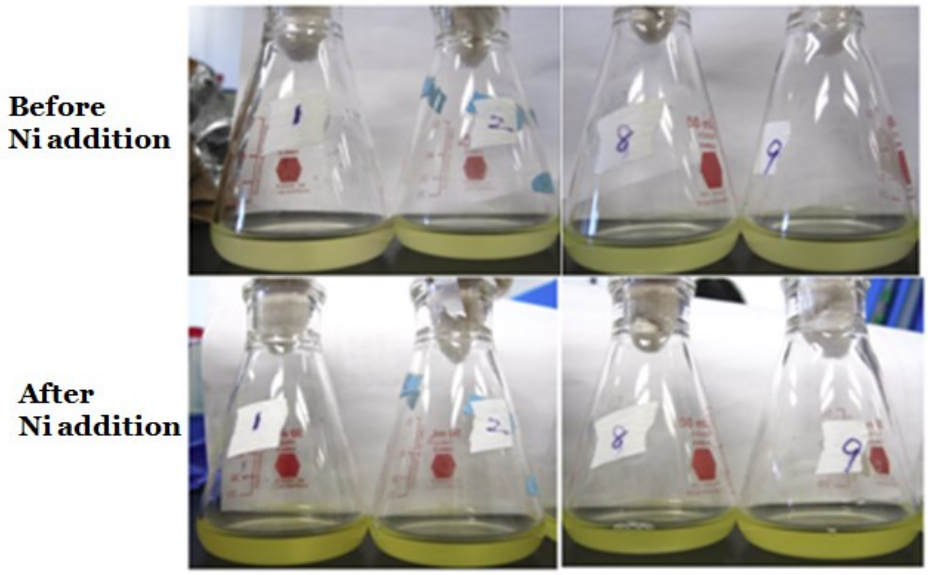

Figure 7. Results of Nickel induction of the *Cyc6: CRLpcr-1-BLE* transformants. Transformants were inoculated in 15 ml medium allowing optimal expression of the *Cyc6* promoter in the dark and induced at 3×10^6 cells/ml by adding 25 μM of $NiSO_4$ for 24 hours.

3.5 Chlorophyll Measurements

In order to detect if any chlorophyll was produced during the nickel induction, five samples were collected from the culture in the dark at the time of 0, 1, 2, 4 and 7 hours after the nickel was added in to the culture, and were measured for chlorophyll concentration. After the first measurement the cultures were transferred under the dim ligh, and for each sample, additional measurements were made at the time of 1, 5 and 24 hours after the first-time measurement. This time-course measurement would indicate the tendency of the chlorophyll changes during the induction. As shown in Figure 8, the value of chlorophyll concentration at each given time (0, 1, 5, 24 hours) is close to zero μg/ml, while the chlorophyll concentration of wild-type strain $4A^+$ is 28.75 μg/ml when the cell density reaches 3×10^6 cells/ml. Thus, compared to the chlorophyll concentration

of the wild-type, the result suggests that chlorophyll was barely produced during the nickel induction.

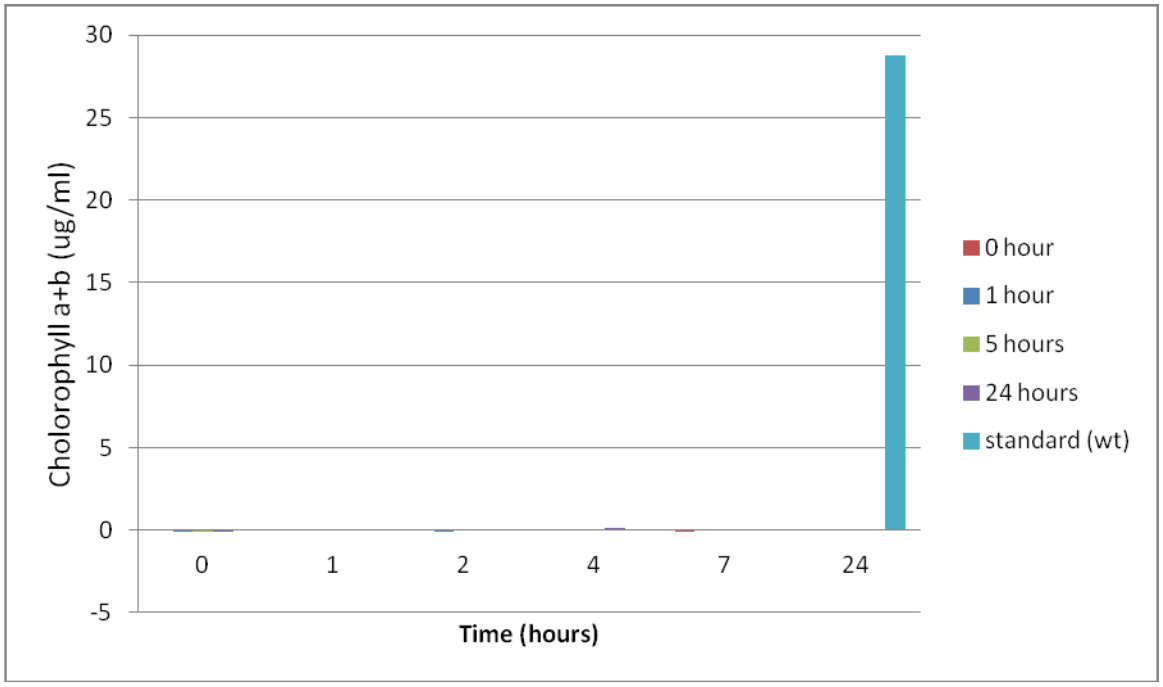

Figure 8. Concentration of chlorophyll (a+b) in transformants treated with 25 μM $NiSO_4$. Samples were collected and measured at the time of 0, 1, 2, 4 and 7 hours after the nickel was added, additional measurements were made at the time of 1, 5 and 24 hours after the first-time measurement. A standard bar shows that the chlorophyll concentration of wild-type strain $4A^+$ is 28.75 μg/ml when the cell density reaches 3×10^6 cells/ml.

3.6 Troubleshooting

Several experiments were performed in order to find out the reasons that cause the failure of producing chlorophyll in our assay.

3.6.1 DNA sequencing

The 4,802 bp chimeric construct was sequenced in order to determine if any mutation occurred during the processes of the PCR or gel purification. The *Cyc6: CRLpcr-1-BLE* fusion construct has the same sequence as the one from the amplifying template, thus, no mutation occurred during the PCR or gel-purification process. The DNA sequence was shown in Appendix.

3.6.2 RT-PCR

RT-PCR was performed to detect if the *CRLpcr-1* gene was expressed driven by nickel inducible *Cyc6* promoter. mRNAs were extracted from the cells treated by $NiSO_4$ (50 μM) for 24 hours. A reverse primer 146-bp downstream from the translational start site was chosen for RT-PCR. The 167 bp PCR fragments amplified from first-strand cDNA in both Ni-inducible cells treated with $NiSO_4$ and mutant cells treated with $NiSO_4$ indicate that the *CRLpcr-1* gene expressed in the mutant *C. reinhardtii* cells (Figure 9). However, from this result it was not possible to conclude that the transgene gene was expressed driven by the *Cyc6* promoter during nickel addition, because the *CRLpcr-1* gene is an endogenous gene which could also express in the mutant strains without the nickel treatment. Thus, the reverse primer located in the *CRLpcr-1* gene region is not enough to detect the transcriptional activity of the *Cyc6: CRLpcr-1-BLE* fusion construct.

Then, another reverse primer very close to the upstream of the start codon of the *CRLpcr-1* gene but located in the *Cyc6* promoter sequence was utilized for RT-PCR, and a 62 bp PCR fragment was expected to see in the Ni-inducible cells, but not in the mutant cells treated with $NiSO_4$. However, no PCR fragment was amplified from the first-strand cDNA in both strains (data not shown). The results indicate that the transgene, the *CRLpcr-1* gene, might not express in transformants during nickel addition. Or the RT-PCR products cannot be detected on an agarose gel by ethidium bromide staining due to the too low amount and too small size of PCR fragment to be detected. Thus, more sensitive method real-time RT PCR can be applied to detect the transcriptional activity.

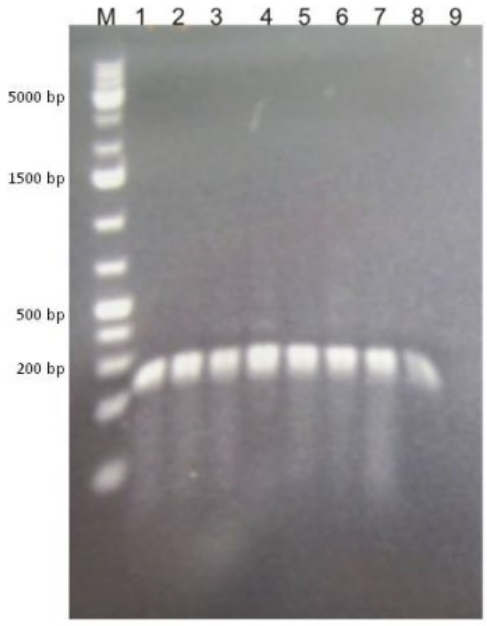

Figure 9. Agarose gel electrophoresis analyses of the RT-PCR fragments (167 bp) from nickel induction of the transformants. The mutant strain #6 treated with 25 μM nickel. Lane 1-7 indicates the transformants treated with Ni; Lane 8 indicates the non-transformants (mutant cells) treated with nickel; Lane 9 indicates the negative control for the RT-PCR, PCR reaction only containing reverse and reverse primers, PCR buffer, DNA polymerase, and dNTPs.

3.6.3 Strain CC-3964 Transformation

Based upon results described above, the fusion *Cyc6: CRLpcr-1-BLE* construct did not properly integrate in and express in the mutant *C. reinhardtii* cells which are sensitive to light. These mutant strains have been maintained for several years for experiment purpose, whether any other mutation happened in the upstream of the chlorophyll pathway or not is not known. Thus, B4 plasmid was transformed in these mutant stains which are expected to see the color changes from yellow to green when these transformants were exposed to the light. This result was obtained from the previous research in our lab several years ago. But unfortunately, such result was not repeated in my experiment. In order test if it is the problem with the strain, a new mutant strain, CC-3964 (*pc1, y7*), was ordered from the *C. reinhardtii* center. This strain has cell wall which has to be removed for transformation, and the strain shows severe palmelloid state, which may affect the transformation efficiency. The cell wall of CC-3964 was digested using gamete autolysin for transformation and the new strain exhibited a really low transformation efficiency compared to the 6 mutant strains (*pc1, y7, cw15, arg7*). After optimizing the possible factors which affect the transformation efficiency, such as palmelloid state in cell cultures, autolysin treatment, only one colony was obtained from the transformants with B4 plasmids.

3.7 DNA Assembler Methods for assembling the *PSBP9-BLE* and the *XRN1-BLE* fusion constructs

The *XRN1* and *PSBP9* genes with unknown function are considered be the candidates involving translation of chloroplast *psbC* mRNA of subunit of PSII complex

(Rochaix, Kuchka et al. 1989). To identify these two genes in *C. reinhardtii*, we used DNA assembler method to create two constructs of *TBC1* genes including *XRN1* and *PSBP9* genes fused with selectable marker *BLE* gene, respectively. The *PSBP9-BLE* and the *XRN1-BLE* fusion constructs were then transformed into the *tbc-1* mutant strain which is PSII deficiency (Bennoun, Masson et al. 1980).

In the *PSBP9-BLE* construct**,** the 5' end of the *PSBP9* gene and the 3' end of the *BLE* gene were designed to overlap with the vector, and there is 50 bp overlapping region between the *PSBP9* and the *BLE* gene. As shown in Figure 10, the PCR products of the expected sizes were obtained for the *PSBP 9* gene (3,578 bp), and the *BLE* gene (1,229 bp) in Lane 1-3.

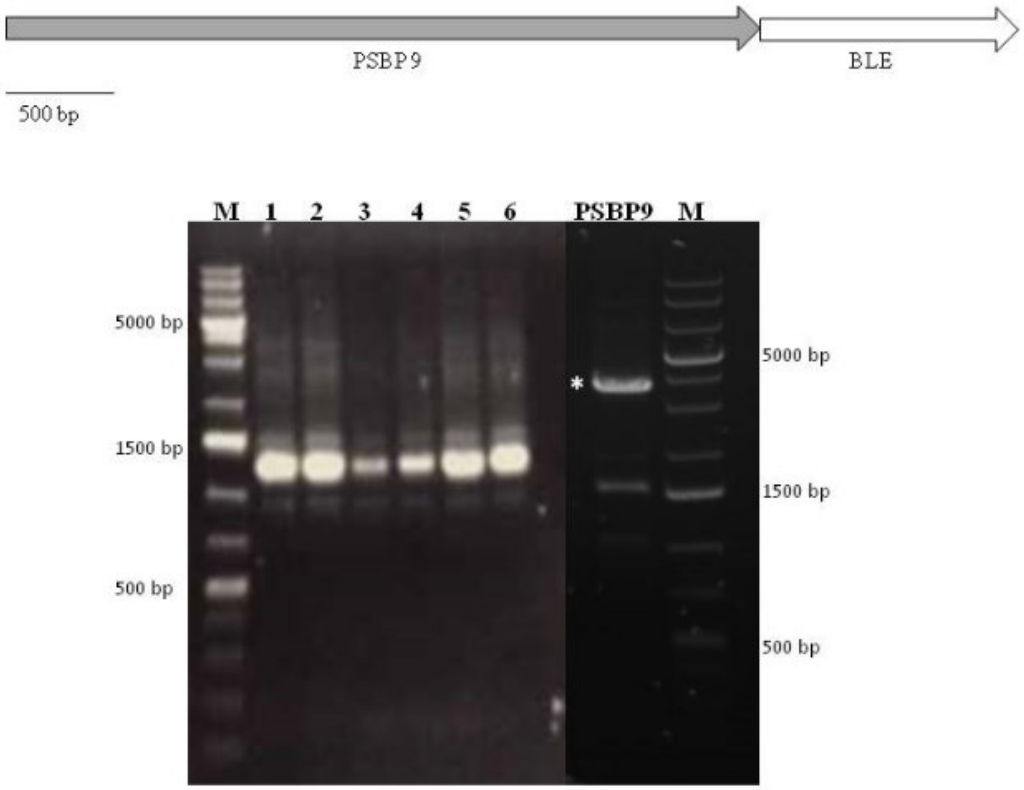

Figure 10. Agarose gel electrophoresis analyses of PCR fragments for constructing the *PSBP9-BLE* fusion construct. Lane 1-3 present the selectable maker of the BLE gene (1,229), M presents the 1 kb plus marker (Fermentas). Symbol "*" indicates the specific PCR fragment.

Due to the large size of the *XRN1* gene (10.3 kb), the whole *XRN1* gene was divided by 6 fragments for DNA assembling. These 6 PCR fragments whose sizes correspondingly are 2,279 bp, 2,372 bp, 1,816 bp, 1,227 bp, 1,587bp and 1,453 bp were shown in Lane C1-C6 in Figure 11. And the selectable marker of the *BLE* PCR fragment was shown in lane 4-5 in Figure 10.

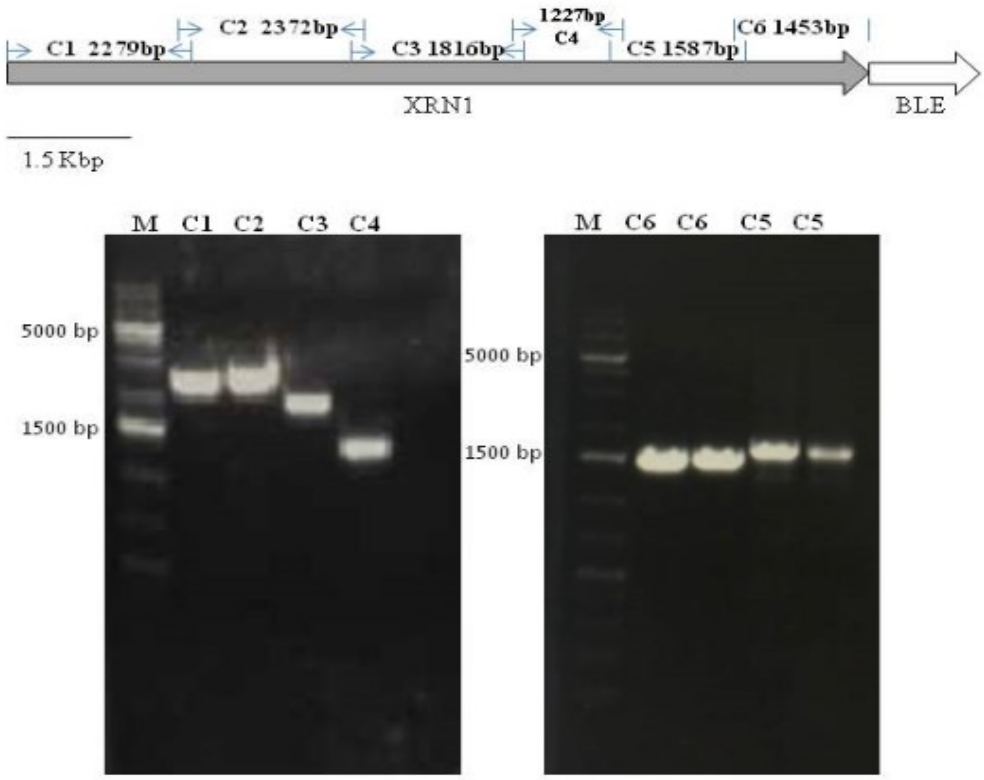

Figure 11. Agarose gel electrophoresis analyses of 6 PCR fragments for constructing the *XRN1-BLE* fusion construct. M presents the 1 kb plus marker (Fermentas).

The *PSBP9* gene together with the *BLE* PCR fragments and the 6 *XRN1* PCR fragments as well as the *BLE* gene were separately transformed into *S. cerevisiae* cells for DNA assembly. The *PSBP9-BLE* fusion construct was confirmed by PCR using the primers flanking at the both end of the whole construct with expected size (4,757 bp) as shown in Figure 12. And the *XRN1-BLE* fusion construct was confirmed by double digestion of the assembled plasmid (data not shown).

Figure 12. Agarose gel electrophoresis analyses of PCR fragments for confirming the *PSBP9-BLE* fusion construct. M presents 1 kb plus marker (Fermentas).

The *tbc-1* mutant which is PSII deficiency in light-dependent photosynthetic pathway can survive using light-independent reaction of photosynthesis (Bennoun, Masson et al. 1980). The mutant strain usually maintained under the dim-light was tranferred on the HSM (High Salt Minimal) plates under the light to test if the mutant strain was revertant. The non-reverting strain should grow neither under the light, nor on the HSM plates.

Both the *PSBP9-BLE* and the *XRN1-BLE* fusion constructs were transformed into the *tbc-1* mutant cells using glass-bead method. The transformants were selected on the zeocin plates and were then tested on HSM plates under the light. The transformants would expect to grow on the HSM plates under the light if the candidate genes are involved in promoting translation of the subunit of PSII complex, since the candidate genes can complement the PSII deficiency in the mutant strains. Around 40 *PSBP9-BLE*

transformats were obtained from the glass-bead transformation, but none of them grew on the HSM plates under the light. And also, I did not get any transformant from *XRN1-BLE* transformation.

Discussion

The industrial and commercial use of ENP products is now leading to the rapid development of the innovations in nanotechnologies for cleaning up industrial contamination, detecting and quantifying the concentration of the nanoparticles in the environment, and improving their productions of manufacturing, delivery, use, and disposal (Seaton, Tran et al. 2010). Due to their extreme small size, EPNs possess special physiochemical and biological properties that the larger particles and their constituents do not have (Steichen, Caldorera-Moore et al. 2012). Researchers are developing new methods to understand the fate of EPNs in the environment and their unknown impacts on the health of humans and other organisms, and ecosystems (Wiesner, Lowry et al. 2006). In our collaborative project, we attempted to develop a method to detect and quantify ENPs in the industrial and receiving environment using biological responses by *C. reinhardtii*. This unicellular green alga is an experimental organism for a variety of problems, including as a model aquatic organism for studies of ecotoxicology. The goals of this project were to understand (1) the potential toxicity of ENPs, (2) minimum concentrations that elicit a response in a living cell, (3) the bioavailable concentration of ENPs in the environment, (4) and whether the toxic effect is caused by nanotoxicity or by their dissolution.

Efficiency of transgene expression in *Chlamydomonas* cells

In our experiment, the fusion construct barely expressed functional protein to complement the mutants. Several possible explanations could account for this result. First of all, in the transcriptional level, the *Cyc6* promoter is a weak promoter in artificial

chimerical gene constructs such that it may not suffice to drive expression of *CRLpcr-1* under the nickel induction condition. In the first example of the use of the *Cyc6* promoter to perform the metal-regulated complementation of a *Chlamydomonas* mutant, the author fused the *Cyc6* promoter to the structural gene of *RSP3*, which encodes radial spoke protein mainly function in regulating axonemal motility and dynein activity of flagella to complement a paralyzed flagella mutant. In their western blot results, the *Cyc6* promoter could only drive much lower levels of *RSP3* protein expression under the nickel induction condition than the constitutive *PSAD* promoter did (Ferrante, Diener et al. 2011). The similar situation is also observed in the study of Surzycki, R. (2007) who fused the *Cyc6* promoter to the coding region of *NAC2,* a nuclear gene which encodes a chloroplast protein required for the stable accumulation of the chloroplast *psbD* RNA. This established the first inducible chloroplast gene expression system. Although, the fusion construct can complement the defects of *nac2* mutant cells, the low amount of this protein was detected in the immunoblot compared to the wild-type protein produced in the wild-type cells (Surzycki, Cournac et al. 2007). Secondly, although, the transformation techniques are being developed for obtaining clones at high frequency, it has been difficult to identify clones that express gene of interest at reasonably high levels (Fuhrmann, Oertel et al. 1999, Schroda, Blocker et al. 2000), which might reflect position effects of genomic regions neighboring the transgene insertion site. In the two studies mentioned above, about 5 out of 68 of transformants showed cells with restored motility after induction with nickel in the complementation of a paralyzed flagella mutant. Of the 55 transformants obtained in the first project, only two showed the expected *Nac2*-dependent expression induced by copper depletion (Ferrante, Diener et al. 2011, Surzycki, Cournac et al. 2007).

The transcriptional mechanisms for the low level of transgene expression in *Chlamydomonas* have not been understood well today. However, the improvements, such as the use of specialized promoters and adjustment of codon usage adapted to high GC content of the *Chlamydomonas* nuclear genome, have been attempted to make for solving this problem which hampered the further functional genomics researches in *Chlamydomonas* (Fuhrmann, Hausherr et al. 2004). Moreover, the post-transcriptional mechanisms can also explain the poor level of gene expression, such as low mRNA stability or poor translation (Wu-Scharf, Jeong et al. 2000, Cerutti and Casas-Mollano 2006).

Transgene Silencing in *C. reinhardtii*

On the other hand, the poor level of transgene expression in *Chlamydomonas* cells is a well known phenomenon, which has been attributed to gene silencing not only for exogenous genes, but might also for endogenous genes (Neupert, Karcher et al. 2009). In a study by the laboratory of Ralph Bock (2009), a new genetic screen method was employed and two *Chlamydomonas* strains that efficiently express the transgene were isolated. They proposed that gene(s) involving epigenetic transgene suppression mechanism has(ve) been knocked out in these strains. Their current unpublished results reveal that it would have been impossible to get the *PCR-1* gene to express under a NP-regulated promoter in the quadruple mutant that was constructed for this project (the *pc1, y7, cw15, arg7* mutant). I was unable to use these strains because they lack flagella and do not cross. Crossing would have been necessary to introduce the *pc1* and *y7* mutations into a strain carrying one of these silencing mutations.

Transformation efficiency

In my transformation experiments, the efficiency of the quadruple mutant strain was extremely low compared to that of the wild-type strains. The positive control of the transformation indicates the low transformation efficiency is not due to the manipulation errors, but may due to the nature of the mutant strains themselves. This could have two explanations. First, the quadruple mutant showed slow growth and is highly light-sensitive. Second, the silencing-defective mutant strains described above showed that the transformation frequencies are correlated with capability of the strains to express transgenes (Neupert, Karcher et al. 2009). Highly gene expression strains increased transformation efficiencies. In agreement with our transformation results, the quadruple mutant recipient strain has both the low expression of the transgene and low transformation frequencies. Both factors prevented the success of this project. Moreover, the mutant cells we used are sensitive to light. The cells exposed to indirect light during transformation procedures could have affected their viability. This factor probably also contributed to the extremely low transformation frequency. With the limited numbers of the transformants obtained from multiple trials, it was not possible to get sufficient numbers of transformants to isolated the correct clone expressing the *Cyc6:CRLpcr-1* transgene, based on the frequency of obtaining the desired transformants expressing the reporter genes in the previous studies: 3% (Surzycki, Cournac et al. 2007) and 7-8% (Ferrante, Diener et al. 2011).

In summary, the *CRLpcr-1* gene was exploited and expected to be served as a useful and robust reporter gene in *C. reinhardtii*. The failure of expressing the *CRLpcr-1* transgene under the nickel inducible condition may be caused by transcriptional gene

silencing mechanism such as position effect of transgene and the very low transformation frequency obtained with the recipient strain for this system. In this case, the strain that is defective in transcriptional silencing (Neupert, Karcher et al. 2009) could be employed to construct our bioassay. This would require reisolation of the original mutants in a strain with flagella so that genetic crossing is possible. Genetic crosses would be required to generate the mutant with the proper genetic background (*pc1, y7, cw15, arg7, uvm4).*

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Appendix

>The DNA sequence of the *Cyc6: CRLpcr-1-BLE* construct

CTCGAGCAGAGGTTGGGAATCGCTTTGAAAATCCAGCAATCGGGTCTCAGCT
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