Optimization of Transgene Expression in *Chlamydomonas reinhardtii* and its Biotechnological Applications

DISSERTATION

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By

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Abstract

Chlamydomonas offers advantages as a recombinant protein production system most importantly because it possesses the machinery required for post-translational modification of proteins. However transgene expression from the nuclear genome of Chlamydomonas is not yet adequate for industrial applications and requires optimization. With the objective of optimizing transgene expression in Chlamydomonas, we analyzed the effect of seven promoters and three transcription terminator elements on the expression of the reporter gene luciferase. We found that in addition to promoters, terminators can have a strong influence on transgene expression. In fact the psaD terminator was much more influential in improving transgene expression than the best promoter identified in our studies: psaD promoter (psaDP). Overall the psaDP-psaD terminator expression cassette was found to be the best expression cassette for transgene expression in Chlamydomonas. It was also established that the state of the plasmid used for Chlamydomonas transformation has a significant impact on transformation efficiency and the percentage of clones expressing the transgene. Furthermore, transgene expression in Chlamydomonas was found to be fairly stable under selection conditions. The findings of this study can be applied to significantly enhance transgene expression in Chlamydomonas and contribute towards developing Chlamydomonas as a biofactory.
The potential biotechnological applications of Chlamydomonas as a biofactory, a mode for oral delivery of vaccines and as a system to control insect-vectors were also demonstrated. A difficult to express human therapeutic protein, human paraoxonase1 (huPON1) and a bacterial protein, acyl homoserine lactone (AHL) lactonase were successfully expressed in Chlamydomonas and were found to be functional. Expression of AHL lactonase in Chlamydomonas resulted in significant reduction in the activity of AHL mimic compounds secreted by Chlamydomonas, indicating that these compounds were inactivated by AHL lactonase, implying the existence of structural similarity between the mimics secreted by Chlamydomonas and AHLs and their potential pharmaceutical applications in inhibiting bacterial growth. The possible application of Chlamydomonas as a system to deliver vaccines orally to fish, animals and possibly humans was demonstrated by expressing luciferase-infectious hematopoietic necrosis virus (IHNV) glycoprotein (G) fusion protein and subsequent feeding of these transgenic algae to rabbits. Feeding of transgenic algae expressing luciferase-G protein fusion to rabbits led to successful induction of immune response in rabbits against the G protein. These transgenic Chlamydomonas will now be evaluated for their ability to vaccinate trout against IHNV.

Chlamydomonas was also successfully developed into a mosquito larvicide, demonstrating its potential in controlling insect-vectors that feed on micro-organisms including microalgae in aquatic bodies. Feeding transgenic algae expressing dsRNA targeting the 3-hydroxy kynurenine (3HKT) enzyme involved in tryptophan metabolism resulted in up to 58% mortality in Anopheles stephensi larvae.
The studies presented in this thesis emphasize the role of regulatory elements like the transcription terminator in enhancing gene expression in Chlamydomonas. This is expected to help further develop Chlamydomonas as an organism of choice for expression of eukaryotic proteins. Moreover the research presented here underlines the potential applications of transgenic Chlamydomonas as an agent for viral, bacterial and insect control.
Dedication

Dedicated to Maya and Bindu.
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CHAPTER 1: INTRODUCTION

1.1  *Chlamydomonas reinhardtii*: A model organism

The unicellular micro alga *Chlamydomonas reinhardtii* has become a model organism for studying photosynthesis, flagellar structure and assembly, organelle biogenesis and cell-cell interactions during mating (68). It has become the preferred organism to study photosynthesis due to its ability to grow both photosynthetically and nonphotosynthetically on acetate as the carbon source, thus allowing rescue of mutants unable to carry out photosynthesis (68). Its ability to grow nonphotosynthetically, requirement of simple medium, short multiplication time, well characterized genetics and availability of a large collection of mutants has attracted many researchers towards Chlamydomonas. The release of the complete genome sequence (107) and ability to manipulate all the three genomes (nuclear, chloroplast and mitochondria) of Chlamydomonas has generated lot of interest in developing biotechnological applications of Chlamydomonas. Following is a brief introduction about *C. reinhardtii* that is principally based on the review by Dr. Elizabeth Harris (68).

*Chlamydomonas* species are unicellular chlorophyte algae having a distinct cell wall, a single large chloroplast and two anterior flagella. *C. reinhardtii* became the most popular laboratory species of Chlamydomonas primarily because of its ability to grow
nonphotosynthetically utilizing acetate as carbon source. The laboratory strains of *C. reinhardtii* were first isolated by GM Smith from soil samples near Amherst, Massachusetts in 1945. The average size of Chlamydomonas cells is about 10µm, even though significant variation in cell size is observed during cell cycle. The cell wall of Chlamydomonas primarily consists of hydroxyproline-rich glycoproteins and lacks cellulose. Several genes coding for various components of cell wall have been identified and a number of mutants with defects in cell wall assembly have been isolated. These cell wall defective mutants have become a very important tool for genetic manipulation of Chlamydomonas because presence of pores in cell wall allows easy entry and integration of exogenous DNA into nuclear genome thereby increasing transformation efficiency. The observation that the cell wall pores allow release of periplasmic proteins into the medium has also generated a lot of interest in using these cell wall defective mutants for production and secretion of foreign proteins into the medium to facilitate protein purification.

The size of Chlamydomonas nuclear genome is about 1 x 10⁸ base pairs and it is packaged into 16 chromosomes. The nuclear genome is GC rich, with a GC content of 64% (107). Nuclear transformation of Chlamydomonas can be easily performed using either particle bombardment (36, 86), glass beads (85), electroporation (145) or silicon whiskers (47). Integration of the transgene into the nuclear genome occurs via non homologous recombination. To facilitate transgene expression, the transgene is usually flanked by native Chlamydomonas 5’ promoter and 3’ UTR regions. Selection of transformants is usually performed with antibiotic selection markers or by
complementing the Chlamydomonas mutant strains (arginine auxotroph or nitrate reductase mutants) with wild type genes argininosuccinate lyase (ARG7) or nitrate reductase (NIT1).

The nuclear envelope, as found in higher organisms is continuous with the endoplasmic reticulum (ER). However, the ER network is not as extensive as the one found in higher plant cells. Two contractile vacuoles are found at the anterior end of the cell. The mitochondria are distributed throughout the cell. The mitochondrial genome is only 15.8kb in size and contains only few genes. Deletion mutants lacking mitochondrial cob1 gene, that codes for cytochrome b, fail to grow on acetate in dark but can grow photosynthetically. The Chlamydomonas mitochondrial genome can also be manipulated using particle bombardment (130).

Chlamydomonas contains a single large cup-shaped chloroplast that occupies about two thirds of cell volume. Presence of the single large chloroplast has made Chlamydomonas a model organism for studying chloroplast biogenesis and photosynthesis. Due to its ability to grow nonphotosynthetically, Chlamydomonas has been widely used to study the role of D1 and D2 proteins of photosystem II, chlorophyll biosynthesis, genetic dissection of electron transport chain and synthesis and assembly of cytochrome b_{6}/f complex. The 195kb long chloroplast genome has been sequenced. Chlamydomonas chloroplast transformation is performed using particle bombardment and transgenes get integrated into the genome by homologous recombination. In addition to isolating several photosynthetic and chloroplast biogenesis mutants using particle bombardment, several transgenes including a single chain antibody (105) and human
glutamic acid decarboxylase 65 (164) have been expressed in Chlamydomonas chloroplast. An important organelle associated with chloroplast is the eyespot, which is located inside the chloroplast membrane at cell equator. The eyespot is rich in carotenoid pigments and appears as a bright orange spot under light microscope. Mutants lacking eyespot show reduced phototaxis.
Figure 1.1: *Chlamydomonas reinhardtii* cell

A diagrammatic representation of an interphase *C reinhardtii* cell. Adapted from http://i.treehugger.com/images/2007/10/24/chlamydomonas%20genome.jpg
Another characteristic feature of *C. reinhardtii* is the presence of two anterior flagella that are 10 to 12 µm in length. The flagella originate from a pair of basal bodies located below the apical end. *C. reinhardtii* flagellar structure and assembly has been studied extensively and large numbers of mutants with defects in motility have been isolated. More than 40 genes coding for flagella components have been cloned and sequenced in *C. reinhardtii*. Homolog’s of some of these genes have been identified in animals (120-122).

*C. reinhardtii* life cycle consists of very distinct mitotic and meiotic phases. Normally the Chlamydomonas cells exist as haploids belonging to two different mating types, plus (mt⁺) and minus (mt⁻). Only under nitrogen limited conditions, sexual cycle is initiated in Chlamydomonas. Cells belonging to both mating types differentiate into sexual gametes when nitrogen is limited. This is followed by flagellar pairing among plus and minus gametes which ultimately leads to fusion of the two haploid gametes, resulting in formation of diploid zygote. The newly formed zygote sheds the flagella and develops into zygospore with the formation of a hard, impermeable wall. Like bacterial and fungal spores, the Chlamydomonas zygospores can remain viable in soil for many years. Zygospores germinate and undergo meiosis resulting in production of four haploid cells under sufficient nitrogen and light conditions.
Figure 1.2: Life Cycle of *Chlamydomonas reinhardtii*

The sexual life cycle of *Chlamydomonas* depicting both diploid and haploid stages. Adapted from http://www.chlamy.org/images/lifecycle.gif.
1.2 Transgene expression in C. reinhardtii

With the demonstration of transgene expression from the nuclear as well as chloroplast genome and the ability to manipulate mitochondrial genome (36, 68, 86, 130), C. reinhardtii is increasingly being recognized as a potential biofactory for production of biopharmaceuticals. Following is the brief update on transgene expression in Chlamydomonas.

1.2.1 Transgene expression from C. reinhardtii nuclear genome

1.2.1.1 Initial attempts and development of selection markers

C. reinhardtii nuclear transformation involves integration of exogenous DNA into the nuclear genome by nonhomologous recombination. The first reports of stable C. reinhardtii nuclear transformation were published in 1989 when Debuchy et al. (36) and Kindle et al. (86) restored the arginine biosynthesis and nitrate reductase function among mutant strains of C. reinhardtii respectively, with the introduction of respective wild type genes by particle bombardment (36, 86). Since then nuclear transformation of C. reinhardtii has also been performed using glass beads (85), electroporation (145) and silicon whiskers (47). Transformation with linearized plasmid was found to be more efficient (85). Nuclear transformation was initially used to determine the function of Chlamydomonas nuclear genes by creating mutant populations through insertional mutagenesis. Initially the selection markers used for insertional mutagenesis included wild-type genes for complementing metabolic mutants, such as ARG7 (argininosuccinate lyase) (36), NIT-1 (nitrate reductase) (86), NIC-7 (nicotinamide-requiring mutant) (53) and THI-10 (thiamine requiring mutant) (53). Even though these selection markers were
suitable for insertional mutagenesis, their application was limited to the respective mutant strains or involved the additional step of introduction of the mutation into the target strain by sexual crossing. Hence efforts were made to develop selection markers that do not suffer from the above mentioned drawbacks.

Transformation of Chlamydomonas with antibiotic selection marker was first performed by Hall et al. (66), wherein they introduced the neomycin phosphotransferase II (nptII) gene driven by nopaline synthase promoter (NOS) along with NIT1 gene. The transformants were initially selected based on their ability to grow on medium containing nitrate. About 52% of the transformants were found to be resistant to kanamycin, indicating that the nptII gene was also expressed by the transformants and the selection marker was functional in Chlamydomonas. Hence, nptII became the first transgene that was expressed in Chlamydomonas. However, expression of nptII was not detected by immunoblot, indicating poor expression of nptII, perhaps resulting from use of a weak NOS promoter and due to significant differences in the codon usage between nptII coding sequence and Chlamydomonas codon usage. The first use of a dominant selection marker for selecting nuclear transformants directly involved use of bacterial ble gene conferring zeocin resistance (155). The fact that ble gene coding sequence was similar to codon usage of Chlamydomonas, perhaps contributed to successful expression of ble in Chlamydomonas. Since then a number of antibiotic selection markers like aminoglycoside phosphotransferase gene aphVIII, conferring paromomycin resistance (148) and aphVII conferring hygromycin resistance (15) have been developed for selecting Chlamydomonas nuclear transformants. In addition to these bacterial antibiotic
resistant genes, several modified Chlamydomonas genes allow dominant selection. These include the \textit{CRY} gene encoding ribosomal protein \textit{S14}, that confers resistance against translation inhibitors cryptopleurine and emetine (113), the mutant protoporphyrinogen oxidase gene (\textit{PPX1}), conferring resistance against porphyric herbicides (131) and genetically modified acetolactate synthase (\textit{ALS}) conferring resistance against sulfonylurea herbicides (88). All the selection markers that have been employed for Chlamydomonas nuclear transformation are listed in table 1.1.
Table 1.1: List of selection markers used for *Chlamydomonas reinhardtii* nuclear transformation.

<table>
<thead>
<tr>
<th>Selection Marker</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argininosuccinate lyase (<em>ARG7</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(36)</td>
</tr>
<tr>
<td>Nitrate reductase (<em>NIT1</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(86)</td>
</tr>
<tr>
<td>Nicotinamide-requiring (<em>NIC-7</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(53)</td>
</tr>
<tr>
<td>Thiamine requiring (<em>THI-10</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(53)</td>
</tr>
<tr>
<td>Ribosomal protein S14 (<em>CRYI</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(113)</td>
</tr>
<tr>
<td>Protoporphyrinogen oxidase (<em>PPX1</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(131)</td>
</tr>
<tr>
<td>Acetolactate synthase (<em>ALS</em>)</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>(88)</td>
</tr>
<tr>
<td>Neomycin Phosphotransferase (<em>nptII</em>)</td>
<td><em>Escherichia coli</em></td>
<td>(66)</td>
</tr>
<tr>
<td>Bleomycin resistance (<em>ble</em>)</td>
<td><em>Streptocollateichus hindustanus</em></td>
<td>(155)</td>
</tr>
<tr>
<td>Aminoglycoside 3’ adenyl transferase (<em>aadA</em>)</td>
<td><em>Escherichia coli</em></td>
<td>(26)</td>
</tr>
<tr>
<td>Chloramphenicol acetyltransferase (<em>cat</em>)</td>
<td><em>Escherichia coli</em></td>
<td>(159)</td>
</tr>
<tr>
<td>Aminoglycoside phosphotransferase (<em>aphVIII</em>)</td>
<td><em>Streptomyces rimosus</em></td>
<td>(148)</td>
</tr>
<tr>
<td>Aminoglycoside phosphotransferase (<em>aphVII</em>)</td>
<td><em>Streptomyces hygroscopicus</em></td>
<td>(15)</td>
</tr>
</tbody>
</table>
1.2.1.2 Initial hiccups with transgene expression from *C. reinhardtii* nuclear genome and their solutions

As mentioned above, the first transgene expressed in Chlamydomonas was *nptII*. Since then a number of reporters and proteins of potential pharmaceutical values have been expressed in Chlamydomonas. Researchers encountered a number of challenges while trying to express transgenes in Chlamydomonas, such as the differences in codon usage, transgene silencing (26) and poor expression of transgenes driven by heterologous promoters, including the widely used *CaMV35S* and *NOS* promoters (66, 159) that are commonly used for transgene expression in plants. Since the expression of the *ble* gene was driven by the regulatory elements of the *RBCS2* gene from Chlamydomonas, efforts were made to identify more efficient and stronger Chlamydomonas promoters. Use of introns as part of the coding sequences had shown to enhance transgene expression in plants. Hence possibilities of improving transgene expression in Chlamydomonas by employing introns were explored. Introduction of first intron of *RBCS2* gene from Chlamydomonas resulted in significant increase in *ble* expression as shown by the accumulation of mRNA and protein as well as the increase in the number of transformants recovered (98). The *RBCS2* intron1 contains an enhancer element (98) and its splicing perhaps stabilizes the mRNA as observed in plants. Recently Stahlberg et al. (48) found that while introduction of more than one *RBCS2* introns further increased transgene expression (1.4 to 1.8 fold increase when compared to inclusion of only intron1), introduction of three *RBCS2* introns in their natural order resulted in 4.5 fold
increase in transgene expression indicating that the number and order of introns have significant impact on gene expression in Chlamydomonas.

The discovery that a fragment of Chlamydomonas *HSP70A* promoter acts as a transcriptional activator and enhances transgene expression when placed upstream of other promoters (β2-Tubulin, *RBCS2* and *HSP70B* promoters) (143) was of great significance. *HSP70A/RBCS2* fusion was found to be best and since then it has become one of the most preferred promoters for transgene expression in Chlamydomonas. Transformation of Chlamydomonas with aminoglycoside 3’ adenyl transferase (*aadA*) driven by *HSP70A/RBCS2* fusion promoter resulted in 2.5 fold increase in the number of transformants recovered (143) compared to the construct with *aadA* driven by *RBCS2* promoter. The *HSP70A* fragment was later found to reduce the probability of transcriptional silencing (142). During initial attempts of Chlamydomonas nuclear transformation, researchers observed that Chlamydomonas transformation with cDNAs of endogenous genes was less successful when compared to transformation with genomic DNA of respective gene. This observation led to discovery of *psaD*, a gene that does not contain introns, but codes for an abundant protein of Photosystem I. Expression of *psaF* cDNA, driven by *psaD* flanking regions was able to complement the 3bF mutant (cw15-arg7-ΔpsaF), which was earlier possible only by using genomic DNA of *psaF* and not the cDNA, indicating that good levels of transgene expression with cDNA’s can also be obtained in Chlamydomonas. Since then the *psaD* promoter has been widely used for transgene expression in Chlamydomonas.
The second factor limiting transgene expression in Chlamydomonas was codon usage. While the genome of Chlamydomonas is GC rich (107), most of the transgenes, particularly the antibiotic selection markers that were tried initially for selection in Chlamydomonas were AT rich as they came from bacteria. The first successful direct selection of transformants was performed using ble gene whose codon usage was similar to Chlamydomonas. Chlamydomonas shows a significant bias towards GC rich codon usage, with 66%, 48% and 86% of GC content at first, second and third nucleotide positions of codons respectively. The first codon optimized gene to be expressed in Chlamydomonas was GFP (59), which was expressed as fusion protein with ble. This fusion allowed direct selection of transformants expressing ble-GFP fusion protein which was detected in western blots. The GFP fluorescence was seen to be localized to nucleus as ble protein localizes to nucleus. In the same work, Fuhrmann et al showed that fusion of GFP with the Cop gene, coding for opsin protein that is localized to the eyespot resulted in localization of GFP to eyespot. However, even though it was a great step forward, the authors only showed expression of GFP as fusion protein indicating that perhaps there were issues with detection of GFP expression when it was expressed alone. Since then codon optimized Renilla luciferase (58) and Gaussia luciferase (138, 144) have been expressed in Chlamydomonas independently, without fusion with any selection marker or Chlamydomonas genes.

The third factor limiting transgene expression in Chlamydomonas is transgene silencing. Chlamydomonas possesses the RNAi machinery and both transcriptional and post transcriptional gene silencing has been observed in Chlamydomonas. Blankenship
and Kindle (17) failed to observe GUS expression driven by \textit{CabII-I} promoter, including detection of GUS transcripts. Upon analysis of PCR positive clones, they found that the \textit{CabII-I} promoter driving transgene expression in all the clones was highly methylated, pointing towards occurrence of transcriptional gene silencing. Cerutti et al. (26) reported that expression of \textit{aadA} gene was unstable in Chlamydomonas with more than 50% of the clones showing gene silencing. Cerutti et al. did not observe methylation of promoter driving \textit{aadA} expression as reported by Blankenship and Kindle (17), but found that silencing of \textit{aadA} was due to suppression of \textit{aadA} transcription by a reversible epigenetic effect (25). Significant differences in the composition of \textit{aadA} sequence and codon usage of Chlamydomonas were also considered as an important reason for such high frequency of \textit{aadA} silencing. Besides codon optimization, use of silencing inhibitor elements such as the ones found in \textit{HSP70A} promoter region, and other techniques that reduce the differences in the composition of introduced DNA elements and Chlamydomonas genome were tried to overcome transgene silencing. As mentioned earlier codon optimization helped to significantly improve transgene expression. The fragment of \textit{HSP70A} promoter that was found to enhance transgene expression from other promoters was found to contain transcriptional silencing inhibitor elements (142). Schroda et al. (143) found that transformation frequency of Chlamydomonas with \textit{ble} driven by \textit{HSP70A/RBCS2} fusion promoter was significantly higher than \textit{ble} driven by \textit{RBCS2} promoter. Further analysis showed that this difference in transformation efficiency was due to significantly high occurrence of \textit{ble} silencing with \textit{RBCS2-ble} constructs when compared to \textit{HSP70A/RBCS2-ble} construct. While \textit{ble} gene was silenced in 80% of
transformants obtained with RBCS2-ble construct, only 36% of transformants obtained with HSP70A/RBCS2-ble construct showed ble silencing. Two elements within the HSP70A promoter region were found to be responsible for this reduced silencing among HSP70A/RBCS2-ble transformants. Since linearized plasmids were used for Chlamydomonas transformation, Schröda et al. also postulated that perhaps the introduction of vector backbone sequences that had significantly different nucleotide composition when compared to the Chlamydomonas genome promoted silencing of introduced ble gene. This problem of vector backbone mediated transgene silencing can be overcome by excising out the transgene expression cassette from vector backbone before Chlamydomonas transformation. In this work we have shown that nuclear transformation of Chlamydomonas with expression cassette results in 50% higher transformation efficiency when compared to transformation with linearized plasmid.

Another significant progress towards realizing the dream of developing Chlamydomonas as a biofactory was made when Neupert et al. (114) recovered mutant Chlamydomonas lines that allow high levels of transgene expression. They assumed that the problem of poor transgene expression in Chlamydomonas has a genetic basis, and used insertional mutagenesis to recover mutants that allow good expression of transgenes. They recovered two mutants, designated as uvm4 and uvm11, which allow efficient transgene expression from the nuclear genome. These strains allowed accumulation of GFP expressed from nuclear genome of Chlamydomonas to levels as high as 0.2% of the total soluble proteins. These clones also showed 1.5 to 3.0 fold higher transformation efficiency when compared to their parent strain.
1.2.1.3 Foreign proteins expressed from nuclear genome of Chlamydomonas

As described above, in the last twenty years substantial progress has been made with respect to transgene expression from nuclear genome of Chlamydomonas. Findings from all the above mentioned studies have not only enabled expression of antibiotic resistance markers and various reporter proteins, but also several proteins of economic value. The potential of developing Chlamydomonas as an edible vaccine was first demonstrated by Sayre et al. (140) when they showed that recombinant proteins targeted and attached to the cell membrane of Chlamydomonas can function as an immunogen inducing immune response and serve as vaccine. Expression of human glycoprotein erythropoietin (Epo) that stimulates red blood production in humans and is required for treating patients suffering from anemia due to chronic kidney disease, was also shown in Chlamydomonas (48). The Epo protein produced in cell wall mutant strain of Chlamydomonas was targeted for secretion into the media for easy purification. Mothbean Δ¹-pyrroline-5-carboxylate synthetase (P5CS) was also expressed successfully in Chlamydomonas (147). Expression of P5CS was found to be effective in improving bioremediation ability of the microalgae as transgenics were able to sequester 4 fold higher cadmium compared to the wild type strain (147). AHL lactonase enzyme from Bacillus sps, that destroys the gram negative bacterial quorum sensing signal molecules, N-acyl homoserine lactones was successfully expressed in Chlamydomonas and the recombinant protein was found to be functional (128). Expression of these proteins from such diverse sources indicates the potential of Chlamydomonas as recombinant protein
production system. However, much needs to be done to bring recombinant protein expression from Chlamydomonas nuclear genome to economically significant levels.
### Table 1.2: List of recombinant proteins expressed from *Chlamydomonas reinhardtii* nuclear genome

<table>
<thead>
<tr>
<th>Recombinant Proteins expressed</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin Phospotransferase (<em>nptII</em>)</td>
<td>Selection</td>
<td>(66)</td>
</tr>
<tr>
<td>Bleomycin resistance (<em>ble</em>)</td>
<td>Selection</td>
<td>(155)</td>
</tr>
<tr>
<td>Aminoglycoside 3’ adenyl transferase (<em>aadA</em>)</td>
<td>Selection</td>
<td>(26)</td>
</tr>
<tr>
<td>aminoglycoside phosphotransferase (<em>aphVIII</em>)</td>
<td>Selection</td>
<td>(148)</td>
</tr>
<tr>
<td>aminoglycoside phosphotransferase (<em>aphVII</em>)</td>
<td>Selection</td>
<td>(15)</td>
</tr>
<tr>
<td>Chloramphenicol acetyltransferase (<em>cat</em>)</td>
<td>Selection</td>
<td>(159)</td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)</td>
<td>Reporter</td>
<td>(59)</td>
</tr>
<tr>
<td>Renilla luciferase</td>
<td>Reporter</td>
<td>(58)</td>
</tr>
<tr>
<td>Gaussia luciferase</td>
<td>Reporter</td>
<td>(138, 144)</td>
</tr>
<tr>
<td><em>Renibacterium salmoninarum</em> p57</td>
<td>Vaccine</td>
<td>(140, 146)</td>
</tr>
<tr>
<td>Human glycoprotein erythropoietin (<em>Epo</em>)</td>
<td>Therapeutic</td>
<td>(48)</td>
</tr>
<tr>
<td>AHL lactonase</td>
<td>Therapeutic</td>
<td>(128)</td>
</tr>
<tr>
<td>Mothbean Δ¹-pyrroline-5-carboxylate synthetase (<em>P5CS</em>)</td>
<td>Bioremediation</td>
<td>(147)</td>
</tr>
</tbody>
</table>
1.2.2 Transgene expression from Chlamydomonas chloroplast genome

Compared to transgene expression from the nuclear genome of Chlamydomonas, much more progress has been made in transgene expression from Chlamydomonas chloroplast. This is perhaps due to the fact that transgene integration in the chloroplast genome occurs via homologous recombination. Consequently, transgenes can be directed for integration into appropriate sites that allow good transgene expression. In addition, lack of transcriptional and post transcriptional gene silencing machinery in chloroplast also ensures stable and often excellent expression of transgenes from Chlamydomonas chloroplast.

1.2.2.1 Initial attempts of transgene expression from Chlamydomonas chloroplast

Chlamydomonas chloroplast transformation was first reported by Boynton et al. (22) in 1988, when they restored the photosynthetic phenotype of a C. reinhardtii mutant strain by particle bombardments with microprojectiles coated with plasmid carrying the wild type chloroplast gene. This was the first report of plastid transformation. Like the nuclear transformation of Chlamydomonas, chloroplast transformation was initially employed to determine the function of chloroplast genes and their regulation (133). Blowers and co-workers made first attempts to express transgenes in Chlamydomonas chloroplast. They transformed Chlamydomonas with constructs carrying bacterial nptII (18) and β-glucuronidase (GUS) (19) genes under the regulatory control of Chlamydomonas chloroplast promoters. While they observed stable integration of the transgenes into the chloroplast genome and accumulation of transcripts, no accumulation of foreign proteins was detected. Expression of a transgene in Chlamydomonas
chloroplast was first reported by Goldschmidt (65) when they observed the expression of the bacterial *aadA* gene flanked by chloroplast promoter and 3’UTR regions. Even though accumulation of *aadA* protein was not shown by immunoblot analysis, transgenic clones were resistant to spectinomycin and streptomycin and showed *aadA* enzymatic activity. It took another eight years for researchers to show accumulation of foreign proteins in *Chlamydomonas* chloroplasts by immunoblot analysis. While Ishikura et al. (77) demonstrated expression of bacterial GUS gene driven by either *atpA*, *rbcl* or *psbA* promoters, Minko and co-workers (109) showed expression of Renilla luciferase driven by *atpA* promoter in 1999. Since then more work has been done to improve transgene expression in *C. reinhardtii* chloroplast through identification of optimal promoters for transgene expression and codon optimization.

The initial studies to determine the best promoter for transgene expression in *C. reinhardtii* chloroplasts were done by Blowers et al. (19). They compared the endogenous transcript levels of *C. reinhardtii* rubisco large subunit (*rbcL*), ATP synthase α-subunit (*atpA*) and ATP synthase β-subunit (*atpB*) genes with the transcript levels of bacterial GUS gene under the control of promoters of the respective genes. The transcripts levels of GUS under the control of *atpA* and *atpB* promoter were found to be similar to the transcript levels of the respective endogenous genes indicating that the 5’ promoter sequences of *atpA* and *atpB* are sufficient to achieve the expression levels of endogenous *atpA* and *atpB* genes. Dissociation of these promoters from their normal structural gene and their integration into a different location in chloroplast genome did not affect their function, indicating that probably these promoters are not regulated by any remote
elements including their 3’UTRs. On the other hand GUS transcripts under the control of rbcL promoter accumulated only to about 1% level of the endogenous rbcL transcripts, signifying that transcription from rbcL promoter is perhaps regulated by other elements that flank the rbcL expression cassette. It was later shown that sequences downstream of rbcL transcription start site enhance rbcL promoter activity (87). Since then the atpA, atpB and rbcL promoters have been commonly used for transgene expression in C. reinhardtii. Kasai et al. (82) showed that fusion of a portion of protein coding sequence of chloroplast genes rbcL (27 amino acids), psbA (20 amino acids) and atpA (25 amino acids) to GUS coding sequence, driven by respective promoters had a significant impact on improving accumulation of GUS protein. The inclusion of a portion of coding sequences perhaps enhances translation and stabilizes the protein. Analysis of the impact of 5’ and 3’ UTR regions of atpA, rbcL, psbA, psbD and 16SrRNA on transgene expression in C. reinhardtii chloroplast showed that transgene transcript and protein accumulation is primarily determined by the promoter and the 3’UTR has little or no effect (10).

The failure to observe accumulation of foreign proteins during the initial attempts of transgene expression in Chlamydomonas chloroplast can be attributed to the differences in the codon usage between the transgenes and Chlamydomonas chloroplast genes. The Chlamydomonas chloroplast genes show strong bias towards codons with A or U at third position (112). As a result, efforts were made to codon optimize transgenes to improve expression in Chlamydomonas chloroplast. The first transgene codon optimized for chloroplast expression was GFP (56). An 80 fold difference in the
accumulation of GFP protein was observed between transgenics expressing codon optimized GFP and non codon optimized GFP indicating that codon usage plays a critical role in expression of transgenes in chloroplast. In subsequent experiments, codon optimized human antibody and luciferase genes were also expressed in Chlamydomonas chloroplast with similar results indicating that codon optimization is a prerequisite for achieving good expression of heterologous proteins in chloroplast of *C. reinhardtii* (105, 109).

Another important factor that has contributed towards rapid progress in *C. reinhardtii* chloroplast transformation is the creation of several deletion strains of *C. reinhardtii* that lack genes essential for carrying out photosynthesis. These mutant strains are widely used for transformation. Wild-type genes are used as selection markers and transformants are recovered based on the restoration of photosynthetic phenotype. This selection is quite popular among researchers as it does not involve antibiotic selection marker. In addition, the *aadA* gene conferring spectinomycin and streptomycin resistance is also employed for recovering *C. reinhardtii* transformants.

### 1.2.2.2 Expression of therapeutic proteins from Chlamydomonas chloroplast

In addition to selection markers and reporter proteins, several proteins of pharmaceutical value have been expressed in *C. reinhardtii* chloroplast. The potential of Chlamydomonas as a platform for production and delivery of vaccines to animals was first demonstrated by Sayre et al. (140). They expressed the p57 protein from *Renibacterium salmoninarum*, a fish pathogen in *C. reinhardtii* chloroplast and showed that feeding of transgenic algae induced immune response in fish. This generated lot of
interest in developing Chlamydomonas as a system for delivery of vaccines orally to animals and humans and the feasibility of expressing a number of antigenic proteins in C. reinhardtii chloroplast was tested. Sun et al. (158), expressed the foot and mouth disease virus VP1 protein fused with cholera toxin B subunit (CTB) in chloroplast of C. reinhardtii. They reported yields of up to 3% of total soluble protein. The fusion protein exhibited GM1-ganglioside binding and antigenicity for VP1 as well as CTB. With the objective of producing cost effective vaccine for controlling type 1 diabetes, human diabetes associated autoantigen, human glutamic acid decarboxylase (hGAD65) was expressed in chloroplast (164). The purified hGAD65 protein was shown to be recognized by the anti GAD antibodies present in the sera from non obese diabetic mouse (an animal model for human type1 diabetes) indicating the antigenicity of algal derived protein and it’s potential in preventing or delaying onset of type1 diabetes. The most credible evidence for using Chlamydomonas as system for production of and delivery of vaccines was recently demonstrated by Dreesen et al. (46). They expressed Staphylococcus aureus fibronectin-binding domain D2, fused with cholera toxin B subunit (CTB) mucosal adjuvant in C. reinhardtii chloroplast to develop an oral vaccine against Staphylococcus infections. Oral immunization experiments on mouse with transgenic algae showed that the antigen produced in chloroplast was effective in inducing immunity in mouse. While 100% mortality was observed in control mice, up to 80% survival was observed in mice vaccinated with transgenic Chlamydomonas upon injection with lethal dosage of Staphylococcus. The antigenic protein inside the chloroplast of algal cell was found to be protected from proteolysis in stomach when
compared to the purified antigen. More importantly, the algal vaccine was stable for more than 1.5 years at room temperature, when stored as lyophilized product.

Another important milestone in the production of human therapeutic proteins in *C. reinhardtii* chloroplasts was the demonstration of expression and assembly of a large single chain antibody against herpes simplex virus (HSV8-Isc) (105). The antibody produced in chloroplast appears to assemble as a dimer and was functional as it recognized the herpes simplex viral coat protein. The antibody accumulated as soluble protein and no traces of insoluble aggregates were detected in the chloroplast. These results indicate that complex proteins can be expressed in *C. reinhardtii* chloroplast. Even though the chloroplasts lack the machinery for posttranslational modification of proteins, they perhaps do have machinery to guide proper folding of proteins, as observed by dimerization of the HSV8-Isc.

In addition to the above mentioned proteins, a number of other proteins of therapeutic value have been expressed in Chlamydomonas chloroplast. Please refer table 1.3 for the complete list of proteins that have been expressed in *C. reinhardtii* chloroplast.
Table 1.3: List of recombinant proteins expressed in Chlamydomonas chloroplast.

<table>
<thead>
<tr>
<th>Recombinant Proteins expressed</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycoside 3’ adenyl transferase (aadA)</td>
<td>Selection</td>
<td>(65)</td>
</tr>
<tr>
<td>Neomycin phosphotransferase (nptII)</td>
<td>Selection</td>
<td>(12)</td>
</tr>
<tr>
<td>B-Glucuronidase (GUS)</td>
<td>Reporter</td>
<td>(77)</td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)</td>
<td>Reporter</td>
<td>(56)</td>
</tr>
<tr>
<td>Renilla luciferase</td>
<td>Reporter</td>
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<td>(158)</td>
</tr>
<tr>
<td>Large single chain antibody against herpes simplex virus (HSV8-Isc)</td>
<td>Therapeutic</td>
<td>(105)</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>Reporter</td>
<td>(157)</td>
</tr>
<tr>
<td>Human metallothionine-2</td>
<td>Therapeutic</td>
<td>(177)</td>
</tr>
<tr>
<td>Human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)</td>
<td>Therapeutic</td>
<td>(174)</td>
</tr>
<tr>
<td>Lac operon repressor protein (lac I)</td>
<td>Regulator</td>
<td>(83)</td>
</tr>
<tr>
<td>Bovine mammary-associated serum amyloid (M-SAA)</td>
<td>Therapeutic</td>
<td>(101)</td>
</tr>
<tr>
<td>human glutamic acid decarboxylase (hGAD65)</td>
<td>Therapeutic</td>
<td>(164)</td>
</tr>
<tr>
<td>Staphylococcus aureus fibronectin-binding domain D2, fused with cholera toxin B subunit (CTB)</td>
<td>Vaccine</td>
<td>(46)</td>
</tr>
</tbody>
</table>
1.2.2.3 Regulating expression of transgenes in chloroplast

Regulation of transgene expression is required when the transgene product is either toxic or not well tolerated by the host. Expression of many of the C. reinhardtii chloroplast genes is regulated by light either transcriptionally or post transcriptionally. The best example is psbA gene coding for D1 protein of photosystemII whose expression is enhanced in the presence of light. Hence, employing the psbA promoter to drive transgene expression provides at least some control over modulating transgene expression. Systems that provide more effective control/regulation of transgene expression in Chlamydomonas are in development. Kato and co-workers have developed an artificial gene expression regulatory system using the E. coli lac regulation system (83). They modified the rbcL and 16S rRNA promoters to add the lac operator. The lac repressor (lac I) under the control of atpA promoter was also introduced into chloroplast. Even though there was some leaky expression in the absence of the inducer, clear isopropyl-β-D-thiogalactopyranoside (IPTG) dependent induction of transgene expression was observed in the transgenics.

1.3 Prelude to thesis

As described earlier, great progress has been made over the last 20 years in improving transgene expression from C. reinhardtii nuclear as well as chloroplast genome. However, only recombinant protein production from C. reinhardtii chloroplast has only been shown to be of economic significance (101, 140) and more needs to be done to improve recombinant protein expression from nuclear genome to make it commercially viable. We have made an attempt to optimize transgene expression from C.
*C. reinhardtii* nuclear genome by addressing some of the fundamental questions related to transgene expression. A large number of promoters are available today for transgene expression in Chlamydomonas, however no comprehensive study has been conducted to compare and identify the best promoter and transformation system for transgene expression. Similarly no effort has been made to identify the optimal transcription terminator region, which plays an important role in transgene expression by contributing to mRNA stability. We have analyzed the effect of seven different promoters and three different transcription terminators on transgene expression in Chlamydomonas. In addition to this, the factors affecting transformation efficiency and stability of transgene expression were also evaluated.

The findings from the above mentioned work were utilized to express a human protein of therapeutic value, human paraoxonase I (*huPON1*) in Chlamydomonas. A glycoprotein from a virus, infectious hematopoietic necrosis virus (IHNV) that infects salmon and trout was also expressed in Chlamydomonas. The experience gained while optimizing transgene expression in Chlamydomonas and the reports of successful control of plant root knot nematodes using transgenic plants expressing RNAi elements against essential nematode (75, 172) genes led to the development of an RNAi mediated strategy for controlling mosquitoes using transgenic algae. Overall the objectives of this research work were:

1. To optimize transgene expression from nuclear genome of *C. reinhardtii*.

2. To develop *C. reinhardtii* as a system for production of recombinant human protein, *huPON1*.
3. To develop microalgal based oral vaccine for fish against IHNV.

4. To develop *C. reinhardtii* as a mosquito larvicide.
CHAPTER 2: OPTIMIZING TRANSGENE EXPRESSION IN

CHLAMYDOMONAS REINHARDTII

2.1 Introduction

The unicellular microalga *Chlamydomonas reinhardtii* has become the organism of choice for studying photosynthesis, flagellar structure and assembly, organelle biogenesis and cell-cell interactions during mating (68). *Chlamydomonas* can reproduce sexually or asexually and can grow either photoautotrophically, heterotrophically and mixotrophically. Availability of complete genome sequence, a large collection of mutants coupled with the ability to manipulate all three genomes (nuclear, chloroplast and mitochondrial) of *Chlamydomonas* along with its well characterized genetics have generated lot of interest in developing biotechnological applications of *Chlamydomonas* (22, 86, 107, 130). The potential biotechnological applications of *Chlamydomonas* range from production of biofuels, hydrogen, and recombinant proteins, such as biopharmaceuticals.

Even though transgenes can be expressed from either the nuclear, chloroplast or mitochondrial genome of *Chlamydomonas*, only protein production from the single large chloroplast of *Chlamydomonas* has been shown to be of economic significance (101, 140). The ability to direct the site of transgene integration in chloroplast genome through
homologous recombination and lack of mechanisms for transcriptional and post-transcriptional gene silencing in chloroplast contribute towards stable and often excellent levels of transgene expression (101, 105). While proper folding of many recombinant proteins and formation of disulphide bonds has been reported in chloroplasts (9, 101, 105, 152, 164, 166), chloroplasts lack the enzymes and machinery to carry out other important post-translational modification of proteins such as glycosylation, which contributes to stability and often functionality of a protein. Thus the inability of chloroplasts to produce glycosylated proteins is the major drawback of recombinant protein production from chloroplast.

Recombinant protein production from the nuclear genome of Chlamydomonas can overcome the limitation of post-translational modification associated with chloroplast expression. Nuclear transformation of Chlamydomonas can be easily performed using either particle bombardment (36, 86), glass beads (85), electroporation (145) or silicon whiskers (47). Moreover, use of mutant strains lacking intact cell wall permit secretion of the recombinant protein into the culture media allowing easy protein purification thereby further reducing the cost of recombinant protein production. Nuclear expression also offers the flexibility of targeting and attaching the recombinant proteins to cell membrane, where the recombinant protein can function as an immunogen inducing immune response and serve as vaccine (140, 146). However, poor expression of transgenes from the nuclear genome of Chlamydomonas has been the major impediment in developing Chlamydomonas as a biofactory for production of recombinant proteins. The molecular mechanisms limiting transgene expression are not known. Possible
mechanisms include gene silencing, inefficient transcription from heterologous promoters, improper RNA processing, mRNA instability and instability of protein products (17, 26, 66). Although significant improvements have been made in transgene expression by identifying several Chlamydomonas promoters (17, 23, 48, 54, 143), codon optimization of transgenes and inclusion of introns (48, 59, 98, 138), transgene expression levels from nuclear genome of Chlamydomonas are not yet adequate and need to be optimized.

The objective of this study was to optimize transgene expression by addressing some of the fundamental questions related to transgene expression in Chlamydomonas. Even though a large number of gene promoters have been identified for transgene expression in Chlamydomonas, no comprehensive study has been conducted to compare and identify the best gene promoters for transgene expression. Similarly no effort has been made to identify the optimal terminator, which plays an important role in transgene expression by contributing to mRNA stability. We have analyzed the effect of seven different promoters and three different terminator elements on transgene expression in Chlamydomonas. In addition to this, the factors affecting transformation efficiency and stability of transgene expression were also evaluated.

2.2 Materials and Methods

2.2.1 Algal strains and cultural conditions

Chlamydomonas strain CC424 (cw15, arg2, sr-u-2-60 mt-) was obtained from the Chlamydomonas culture collection at Duke University, USA. Strains uvm4 and uvm11 were kindly provided by Dr. R. Bock (Max Planck institute, Germany). Strains were
grown mixotrophically in liquid or on solid TAP (mineral salts + acetate) medium (69) at 23°C under continuous white light (40 µE m⁻² s⁻¹), unless otherwise stated. Medium was supplemented with 100 µg/mL of arginine when required. For selection of transformants solid TAP medium was supplemented with 50 µg/mL of paromomycin and 100 µg/mL arginine. For induction of Feal promoter driven luciferase expression, transformants were first grown in liquid TAP medium till log phase (OD₇₅₀=0.8 to 1.0). Cells were then pelleted, washed once with low iron TAP medium (Fe free TAP supplemented with 1 µM of Fe-EDTA) and resuspended in low iron TAP medium for growth.

2.2.2 Vector Construction

Plasmid pHsp70A/Rbcs2-cGLuc (70) carrying codon optimized Gaussia princeps luciferase for Chlamydomonas nuclear expression was obtained from the Chlamydomonas culture collection at Duke University, USA. Luciferase coding sequence was amplified using primers LUCFwd1 (5' - ATCTACATATGCTCGAGATGGGCGTGAAGG- 3') and LUCRev1 (5' - AAGATAAGCTTCTAGATTACGTATCGCCGAGCG- 3'), introducing NdeI and XbaI sites at 5' and 3' ends, respectively. The Luciferase coding region was cloned as NdeI/XbaI fragment into the similarly digested vector pSSCR7 (147), creating plasmid pCVAC110. The paromomycin expression cassette from vector pSL18 (39) was excised as Xhol/KpnI fragment and cloned into same sites of pBlueScriptKS+ to create plasmid pCVAC112. The B2-Tubulin promoter-luciferase-low carbon dioxide inducible protein (CCP1) terminator cassette from pCVAC110 was then amplified using primers B2TubP Fwd(5' - ATCTAACTAGTCTGCAGCAAGCTGGCACAATTCTTTGTGC - 3') and LCIMP
rev (5’- ATCTTGTCGACAAAGCTGTTCCCTTGTTCCGC- 3’) to introduce PstI and SalI sites at 5’ and 3’ ends respectively and cloned into Xhol/PstI sites of pCVAC112 thereby destroying the Xhol site to create vector pCVAC113. To create additional vectors in which luciferase expression is driven by various promoters, *B2-Tubulin* promoter from pCVAC112 was excised as PstI/NdeI and replaced by the promoter sequences of the Chlamydomonas Fea1, psaD, Hsp70A/Rbcs2P, Chlorella virus DNA polymerase (CVDP), CaMV35S and Chlamydomonas Actin genes creating plasmids pCVAC115, pCVAC117, pCVAC120, pCVAC123, pCVAC129 and pCVAC130 respectively. The primers used to amplify the above mentioned promoters are listed in table1.

Luciferase amplified with primers LUCFwd1 and LUCRev1 was also cloned into NdeI/XbaI sites of vector pSL18 (39) creating plasmid pCVAC157. *B2-Tubulin* was amplified with primers B2TubPFwd1 and B2TubPRev1 from pCVAC113 and cloned into StuI/NdeI site of pCVAC157 replacing psaD promoter, creating plasmid pCVAC167. To amplify and clone *B2-Tubulin* terminator from Chlamydomonas genome, primers B2TubTFwd1 and B2TubTRev1 were used. The *psaD* terminator from vectors pCVAC167 and pCVAC157 was excised as XbaI/NotI fragment and replaced with *B2-Tubulin* terminator to create plasmids pCVAC168 and pCVAC169 respectively. Plasmid pCVAC164, carrying two copies of *psaD* 3’ terminator was constructed by amplifying the 1kb luciferase-*psaD*3’ terminator region from plasmid pCVAC157 using primers LucFwd1 and *psaD*3’Rev and cloned into NdeI/EcoRI sites of pSL18. Refer to table 2.1 for sequence of primers used for cloning *B2-Tubulin* and *B2-Tubulin* terminator and luciferase-*psaD*3’ terminator.
The consensus nucleic acid binding protein1 (NAB1) binding site from Chlamydomonas LHCII isoforms (110) was fused to the luciferase coding sequence following the start codon using primer LUCFwd2. Luciferase was amplified with primers LUCFwd2 and LUCRev1 and cloned into NdeI/XbaI sites of vector pSL18 creating plasmid pCVAC105. Similarly the mutantNAB1 site (coding for the same six amino acids coded by the NAB1 binding site, but differs in nucleotide composition from the NAB1 binding site) was fused to the luciferase coding sequence following the start codon with primer LUCFwd4. Luciferase amplified with primers LUCFwd4 and LUCRev1 was cloned into NdeI/XbaI sites of pSL18 vector creating plasmid pCVAC133. NAB1 binding site was fused nine bases following the luciferase stop codon using primer LUC3’NAB1. Luciferase amplified with primers LUCFwd1 and LUC3’NAB1 was restriction digested with enzymes NdeI and XbaI and cloned into same sites of pSL18 to create plasmid pCVAC163. For sequences of primer LUCFwd2, LUCFwd4 and LUC3’NAB1, refer table 2.1.

2.2.3 Nuclear transformation of *Chlamydomonas reinhardtii*

*C. reinhardtii* nuclear transformation was performed using the glass beads method (85). Briefly, CC424 strain of Chlamydomonas was grown in 100 mL of TAP liquid media supplemented with arginine. Cells were harvested in log phase (OD\textsubscript{750}=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 4 mL of sterile TAP + 40 μM sucrose. 300 μL of cells were transferred to a sterile microcentrifuge tube containing 300 mg of sterile glass beads (0.425-0.6mm, Sigma, Saint Louis, USA). Sterile 20% PEG 6000 (100 μL) (Sigma, Saint Louis, MO, USA) was added to the cells along with 1.5 μg of plasmid
DNA. Prior to transformation, all constructs were restriction digested either to linearize the construct or to excise the two expression cassettes carrying selection marker and gene of interest together, from the plasmid backbone. Following addition of plasmid DNA, cells were vortexed for 20 seconds and plated on to TAP agar plates containing 50 µg/mL paromomycin and 100 µg/mL arginine.

In order to compare the efficiency of transformation with supercoiled plasmid, linearized plasmid or excised expression cassette, 1x10⁷ cells of Chlamydomonas were transformed with 1 µg of plasmid DNA in each experiment.

2.2.4 Luciferase assays

Luciferase assays were performed at room temperature with POLARstar OPTIMA microplate luminometer (BMG Labtech, Germany) with automated substrate injection. For screening Chlamydomonas transformants for luciferase expression, transformants were inoculated in 2 mL 96 well (Nunc, Rochester, NY, USA) plates containing TAP media supplemented with arginine and grown for 2 to 3 days under continuous light. One hundred microliters of algal culture was diluted with 100 µL of TAP medium in 96 well plate and luminescence was recorded following automatic injection of 10 µL coelenterazine (0.1 mM in 10% ethanol) (NanoLight technologies, Pinetop, AZ USA). Luminescence was recorded every 4 s for total of 24 s at the highest amplification and directly referred to as relative luminescent units.

For calculating relative luminescent units (RLU), luciferase activity of each culture was normalized to OD₇₅₀ of the culture. Secretion of luciferase into the culture medium was confirmed by measuring luciferase activity in culture supernatant. One
hundred micro liters of culture supernatant obtained following centrifugation at 12,000 rpm was mixed with 100 µL of liquid TAP medium in a 96 well plate for measuring luminescence. TAP medium (200 µL), and culture supernatant from nontransgenic strain were included as controls.

2.2.5 Genomic DNA isolation and Southern blot analysis

Chlamydomonas genomic DNA was isolated from 1.0 x 10^7 cells using a Qiagen Plant DNAeasy kit (Qiagen Inc., Valencia, CA, USA) following manufacturer’s protocol. For Southern blot 5.0 µg of genomic DNA was used. Genomic DNA from CC424/pCVAC117 and CC424/pCVAC157 clones was digested with SacI and XhoI respectively at 37°C for 16 hours and separated on 1% agarose gel in Tris-Borate-EDTA buffer (89 mM tris base, 89 mM boric acid and 2 mM EDTA). Following acid hydrolysis with 0.25N HCL for 15 minutes, gel was neutralized by treating with 0.4M NaOH/0.6M NaCl and 1.5M NaCl/0.6M tris (pH7.5) for 15 minutes. Gel was then equilibrated in 10X sodium chloride-sodium citrate (SSC) buffer (3M NaCl, 300 mM sodium citrate, pH 7.0) and capillary transfer with 10X SSC was set to transfer DNA samples to a positively charged nylon-66 membrane. DNA fragments were cross-linked by UV-irradiation in a Stratalinker® (Stratagene, La Jolla, CA, USA) by using autocrosslinking setting. Following UV crosslinking, membranes were rinsed briefly in double distilled water and pre-hybridized with DIG-Easy-Hyb solution (Roche, Mannheim, Germany) at 40°C for 60 min. Heat-denatured, DIG labeled Gaussia luciferase probe made using luciferase PCR product as template with the DIG-High Prime kit (Roche, Mannheim, Germany) was used for overnight hybridization at 50°C in DIG-Easy-Hyb solution. Detection was
completed with the DIG Wash and Block Buffer set (Roche) and the anti-digoxigenin (DIG)-alkaline phosphatase conjugate antibody following manufacturer’s protocol.
Table 2.1: Sequences of primers used for amplifying and cloning luciferase, various promoter and 3’UTR elements used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fea1Fwd</strong></td>
<td>5’- ATCTAACTAGTCTGCAGTACCAGGACAGAGTGCGTGTGG-3’</td>
</tr>
<tr>
<td><strong>Fea1Rev</strong></td>
<td>5’- GAAATCATATATGCTCGAGTTGGCCTGTGTAGAAAGTG-3’</td>
</tr>
<tr>
<td><strong>Hsp70A/Rbcs2Fwd</strong></td>
<td>5’- ATCTAACTAGTCTGCAGGAGCTCGCTGAGGCTTGACATG-3’</td>
</tr>
<tr>
<td><strong>Hsp70A/Rbcs2Rev</strong></td>
<td>5’- TAGAACATATGCTGCAATGGAAACCGCGACG-3’</td>
</tr>
<tr>
<td><strong>psaDFwd</strong></td>
<td>5’- ATTCGCTGCACACACACCTGCCCCTCG-3’</td>
</tr>
<tr>
<td><strong>psaDRev</strong></td>
<td>5’- AGGATCACTATGGGCTTGTGTAGTAGCAGAGTG-3’</td>
</tr>
<tr>
<td><strong>DPolFwd</strong></td>
<td>5’- ATCTCTGCAGCAAAGAACGTGACTCTTG-3’</td>
</tr>
<tr>
<td><strong>DPolRev</strong></td>
<td>5’- AGAATATTTAATTCATTGTATTTCATTATGATACATAG-3’</td>
</tr>
<tr>
<td><strong>CaMV35SFwd</strong></td>
<td>5’- ATCGATCTGCAGATGGTGAGCACGAGAC-3’</td>
</tr>
<tr>
<td><strong>CaMV35SRev</strong></td>
<td>5’- ATGCTCATATGAGAGATAGATTTGTAG-3’</td>
</tr>
<tr>
<td><strong>ActinFwd</strong></td>
<td>5’- ATCTTCTGCAGAGGTGCATGCGCTCCACGATTAG-3’</td>
</tr>
<tr>
<td><strong>ActinRev</strong></td>
<td>5’- AAGATCATATGTTTGAATCCTGCGTGACTGC-3’</td>
</tr>
<tr>
<td><strong>B2TubPFwd1</strong></td>
<td>5’- ATCTTAGGCTTCTTGCTGCTATGACCTCAAAGTGC-3’</td>
</tr>
<tr>
<td><strong>B2TubPRev1</strong></td>
<td>5’- TAATAGCATATGAGTGCTGCTTGTGTAAGTAGTG-3’</td>
</tr>
<tr>
<td><strong>B2TubTFwd1</strong></td>
<td>5’- CTATTATCTAGATGCAGCCGACCTTACGTC-3’</td>
</tr>
<tr>
<td><strong>B2TubTrev1</strong></td>
<td>5’- AAGATGCGGCCGCGGTATATCTATTACAGTACGACTGGAATG-3’</td>
</tr>
<tr>
<td><strong>LUCFwd2</strong></td>
<td>5’- ATCTTCATATGTGCAGACCGGGCAGGCCGCTCGAGATGGGAGG-3’</td>
</tr>
<tr>
<td><strong>LUCFwd4</strong></td>
<td>5’- ATCTTCATATGTGTAACACACCCGCAGGGGGGTGCTCGAGATGGGAGG-3’</td>
</tr>
<tr>
<td><strong>LUC3’NAB1</strong></td>
<td>5’- TAGATTCCTAGACCCGCGGGGCTGTCGATAATATTATTACGTATCGCGCAGCG-3’</td>
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</tbody>
</table>
2.2.6 Real Time PCR

Total cellular RNA was extracted from 1.0 x 10^7 cells using Nucleospin RNAII kit (Clontech, Mountain view, CA, USA) according to manufacturer’s instructions. Contaminating genomic DNA was removed by treating RNA samples with DNAase I (Promega, Madison, WI USA) following manufacturer’s instructions. RNA quality and concentration was measured using Nanodrop (Thermo-scientific, Wilmington, DE, USA). Structural integrity of the RNAs was checked with non-denaturing agarose gel and ethidium bromide staining. 2.0 µg of total RNA was used to setup cDNA synthesis using Quantas cDNA synthesis mix (Quantas, USA) following manufacturer’s instructions. Real-time quantitative RT-PCR was carried out using an ABI – Step One Plus (Applied Biosystems, Foster City, CA, USA) using PerfeCTa™ SYBR® Green FastMix™ (ROX dye) (Quanta Biosciences, Gaithersburg, MD, USA) according to manufacturer’s instructions. The Chlamydomonas CβLP gene (CβLP Fwd 5’- AGTGCAAGTACACCATGGCGA - 3’ and CβLP Rev 5’- CTTCAGCTTGCTTGAGTCAGGTT - 3’) was used as reference gene/internal control and was amplified in parallel with the target Luciferase gene (LUCRealFwd 5’- CACGCCCAGATGAAGAAGTTCATTCC- 3’ and LUCRealRev 5’- CTTCTTGAGCAGGTCGGAAACTG- 3’) allowing gene expression normalization and providing quantification. Reactions were carried out with 10 ng of cDNA. All the primers were designed using the Primer Express software following the manufacturer’s guidelines. (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For each sample, reactions were set up in quadruplicates and two biological experiments
were done to ensure the reproducibility of the results. The quantification of the relative transcript levels was performed using the comparative $C_T$ (threshold cycle) method (96).

2.2.7 Western blot analysis

Cells equivalent to one milliliters of $\text{OD}_{750}=1$ from algal cultures were pelleted in microcentrifuge tubes by centrifugation at 10,000 rpm for 1 minute at room temperature and resuspended in 50 μL of 1X Laemmlii loading dye (0.063M Tris-HCl Ph 6.8, 2% SDS-PAGE, 10% glycerol, 5% B-mercaptaethanol, 0.05% bromo-phenol-blue), and incubated at 100°C for 5 minutes. Samples were removed from water bath, cooled to room temperature, centrifuged at 10,000 rpm for 2 minutes and the cell extract was separated on 10% SDS polyacrylamide gel by electrophoresis. Protein samples were transferred to PVDF membrane (Millipore, Billerica, MA, USA) by semi-dry blotting. Membrane was incubated with anti G-LUC rabbit polyclonal antibody (Nanolight technologies, Pinetop, AZ, USA) at 1: 1000 dilution at 4°C for overnight following blocking of membrane with 0.5% BSA in TBS containing 0.05% Tween-20. After washing the blot for 3 x 5 minutes with TBST (TBS +0.05% Tween 20), blot was incubated with anti-rabbit IgG antibody (Sigma, Saint Louis, MO, USA) conjugated to horse-radish peroxidase at 1:10000 dilution for 2 hours at room temperature. Following three washes with TBST, detection was completed by chemiluminescence using luminol (0.4 mM) and iodophenol (8.0 mM) (Sigma, Saint Louis, MO, USA) (173).
2.3 Results

2.3.1 Chlamydomonas psaD promoter is an excellent promoter for transgene expression in Chlamydomonas reinhardtii

Seven promoters were evaluated to identify the best promoter for driving transgene expression in Chlamydomonas. Among these seven promoters, five promoters were Chlamydomonas promoters, namely B2-Tubulin, Fea1, psaD, Actin and Hsp70A/Rbcs2 fusion promoter. Two heterologous viral promoters were also evaluated for transgene expression in Chlamydomonas. Since no viruses infecting Chlamydomonas are known, we decided to test a promoter from a virus infecting another unicellular microalga Chlorella (Paramecium bursaria Chlorella virus-1 (PBCV-1), using the Chlorella virus DNA polymerase promoter (CVDP). The CVDP was selected because it has been demonstrated to perform as well as the Cauliflower Mosaic virus 35S (CaMV35S) promoter for driving transgene expression in Chlorella (79). The most preferred promoter for transgene expression in plants, the CaMV35S promoter was also tested. With the exception of the Fea1 promoter which is induced under iron deficiency all other Chlamydomonas promoters tested in this study are constitutive promoters. The Hsp70A/Rbcs2 and Actin promoters harbor an intron in their 5’UTR region that is known to enhance the gene expression. Except for Fea1, Actin and CVDP promoters, all other promoters have been used for transgene expression in Chlamydomonas, but no comprehensive study has been done to compare and identify the best promoter for transgene expression in Chlamydomonas. More information about the promoters used is described in table 2.2.
Luciferase from *Gaussia princeps* was used as reporter. Codon optimized *Gaussia* luciferase for *Chlamydomonas* nuclear expression (70) was obtained from the *Chlamydomonas* culture collection at Duke University, USA and the promoter-luciferase constructs were made. All the constructs with different promoters driving luciferase expression were identical in all aspects except for the promoter (Figure 2.1). Each construct was restriction digested either to linearize the plasmid or to excise the expression cassettes carrying selection marker and gene of interest together, from the plasmid backbone before *Chlamydomonas* transformation. For each construct, 83 transformants selected on agar plates carrying paromomycin were screened for luciferase activity. Since the integration of transgene into the nuclear genome of *Chlamydomonas* occurs via nonhomologous recombination, the transformants exhibited a broad range of luciferase activity for each construct. Nine clones showing highest luciferase activity from each construct were selected for carrying out relative luminescent units (RLU) measurement. Luciferase activity exhibited by each culture was measured and normalized to OD\textsubscript{750} of the culture to calculate RLU. The data was analyzed following single factor analysis of variance (ANOVA). Significant differences were observed in luciferase expression levels among clones expressing luciferase under the control of different promoters. Highest expression of luciferase was seen with *psaD* promoter followed by *Actin, Hsp70A/Rbcs2* and *B2-Tubulin* promoters (Figure 2.2). The clones expressing luciferase under the control of the above mentioned promoters differed significantly with respect to luciferase expression levels. The clones expressing luciferase under the control of *Fea1P, CVDP* and *CaMV35SP* did not differ significantly, but had significantly lower
levels of luciferase expression when compared to the other four promoters. Lowest expression was observed with \textit{Fea}1 promoter. Among Chlamydomonas constitutive promoters, lowest expression was observed with \textit{B2-Tubulin} promoter. Luciferase expression with \textit{psaD} promoter was about 85 fold higher than \textit{Fea}1 promoter and about 4.6 fold higher than the weakest Chlamydomonas constitutive promoter, \textit{B2-Tubulin}. The two heterologous viral promoters, \textit{CaMV35S} and \textit{CVDP} also performed poorly. Results of RLU experiment were confirmed by checking the luciferase protein levels in transgenic Chlamydomonas clones by western blot (Figure 2.3).

The nucleotide sequences of Chlamydomonas \textit{B2-Tubulin, psaD, Actin} and \textit{Hsp70A/Rbc}s2 fusion promoters were subjected to plant cis-acting regulatory DNA elements (PLACE) software (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (72) to investigate whether the \textit{psaD} promoter has any unique transcription factor binding sites that perhaps contribute towards its ability to drive excellent transgene expression. However we failed to observe any such unique element in the \textit{psaD} promoter sequence.
Table 2.2: Detailed information regarding the promoters evaluated for transgene expression in *Chlamydomonas reinhardtii*.

<table>
<thead>
<tr>
<th>Promoter name</th>
<th>Promoter Type</th>
<th>Amplified from</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Chlamydomonas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B2-Tubulin</em></td>
<td>Constitutive</td>
<td>pSSCR7</td>
<td>Davies et al. (33)</td>
</tr>
<tr>
<td>Chlamydomonas <em>Fea1</em></td>
<td>Inducible</td>
<td>pJD100</td>
<td>This work</td>
</tr>
<tr>
<td>Chlamydomonas <em>psaD</em></td>
<td>Constitutive</td>
<td>pSL18</td>
<td>Fischer and Rochaix (54)</td>
</tr>
<tr>
<td><em>Hsp70A/Rbcs2fusion</em></td>
<td>Constitutive</td>
<td>pSL18</td>
<td>Schroda et al. (143)</td>
</tr>
<tr>
<td>Chlorella virus DNA Pol</td>
<td>Constitutive</td>
<td>Genomic DNA</td>
<td>This work</td>
</tr>
<tr>
<td><em>CaMV35S</em></td>
<td>Constitutive</td>
<td>pC2301</td>
<td>Tang et al. (159)</td>
</tr>
<tr>
<td>Chlamydomonas <em>Actin</em></td>
<td>Constitutive</td>
<td>Genomic DNA</td>
<td>This work</td>
</tr>
</tbody>
</table>
Figure 2.1: Schematic drawings of all the promoter constructs used to study transgene expression in *Chlamydomonas reinhardtii*.

Schematic drawings of constructs used to compare seven promoters for transgene expression in *Chlamydomonas reinhardtii*. All the constructs are identical in all aspects and differ only with respect to the promoter driving luciferase expression. All the promoters were cloned into the PstI/NdeI sites of vector pCVAC113. All the vectors carry the AphVIII gene as selectable marker that confers resistance to paromomycin and is driven by *Hsp70A/RbcS2* hybrid promoter.
Figure 2.2: Comparison of different promoter constructs by measuring Relative Luminescence Unit (RLU) activity.

Comparison of different promoter constructs by measuring Relative Luminescence Unit (RLU) activity. Nine clones showing highest luciferase activity from each construct were selected for carrying out RLU measurement. RLU was calculated by normalizing the luciferase activity exhibited by each culture to OD$_{750}$ of the culture. The data was analyzed by single factor ANOVA. While the table F value is 2.16, the calculated F value was 29.39 at 1.05 x 10^{-17} level of significance indicating that there are significant differences among the clones expressing luciferase under the control of different promoters. The calculated critical difference (CD) value was 71354. Comparison of different constructs using CD value indicates that clones expressing luciferase under the control of psaDP, ActinP, Hsp70A/Rbcs2P and B2-TubulinP differ significantly with respect to levels of luciferase expression. The clones expressing luciferase under the control of Fea1P, CVDP and CaMV35SP did not differ significantly, but had significantly lower levels of luciferase expression when compared to the other four promoter constructs. N=72
Western blot analysis of Chlamydomonas strains transformed with different promoter constructs (pCVAC117 (psaDP-LUC-CCP1 ter), pCVAC120 (HSP70A/Rbcs2P-LUC-CCP1 ter), pCVAC113 (B2-TubulinP-LUC-CCP1 ter), pCVAC129 (CaMV35SP-LUC-CCP1 ter) and pCVAC130 (ActinP-LUC-CCP1 ter)). Colonies showing highest luciferase expression were selected from each construct for western analysis. Cells equivalent to one milliliters of OD$_{750}$=1 were loaded from each culture. Blot was probed with anti Gausstia luciferase antibody. Dual color precision plus protein ladder was used. The two pink bands in the ladder represent bands of 25 kDa and 75 kDa in size.
2.3.2 psaD terminator significantly improves transgene expression in Chlamydomonas

Most of the research done on optimizing transgene expression in plants and Chlamydomonas has focused on identification of strong promoters to maximize transgene expression as an important strategy, while little effort has been made to identify the optimal transcription terminator to improve transgene expression. A transcription terminator is a sequence downstream from the 3' end of an open reading frame that marks the end of a gene and signals RNA polymerase to release the newly made RNA molecule. Except for few studies in plants that compared various terminators (24, 76, 111), no such study has been conducted with Chlamydomonas. The terminator regulates gene expression by controlling transcription termination and 3’ end processing of mRNA. Proper 3’ end processing is important as it determines stability, nuclear to cytoplasmic export and translation competence of mRNA (178). Importance of a strong terminator signal in gene expression can be gauged from the fact that weak terminator signals allow read through transcription resulting in improper 3’ end processing of mRNA which promotes gene silencing leading to reduced transgene expression (99). In fact transformation of plants with constructs lacking terminator is becoming an important strategy for targeted gene silencing (115).

Hence we decided to test the impact of different terminator signals on expression of luciferase. Terminator regions of CCP1 (125, 129), B2-Tubulin and psaD were selected for this study. Six constructs were made using these three terminator regions in which the luciferase expression was driven by B2-Tubulin and psaD promoters (Figure
2.4). A construct with two copies of psaD terminator was also tested. CC424 strain of Chlamydomonas was transformed with these constructs. Ninety three transformants selected on paromomycin plates were screened for luciferase activity from each construct. Nine clones showing highest luciferase activity were selected from each construct for carrying out RLU assay. The data was analyzed following single factor ANOVA. With the exception of psaDP-LUC-B2-Tubulin terminator and psaDP-LUC-CCP1 terminator constructs that did not differ significantly, all other constructs differed significantly from each other with respect to levels of luciferase expression. Highest luciferase expression was seen with clones carrying constructs with psaD terminator, irrespective of the promoter driving luciferase expression (Figure 2.5). The other two terminators performed poorly with both the promoters. While the B2-Tubulin terminator performed better than CCP1 terminator in combination with B2-Tubulin promoter, CCP1 terminator performed slightly better than B2-Tubulin terminator in combination with psaD promoter. The results clearly indicate that terminators have significant impact on the level of transgene expression in Chlamydomonas. Among clones carrying psaD terminator, highest expression was observed with psaDP-LUC-psaD ter (pCVAC157) construct where luciferase expression was about 6 fold higher than corresponding CCP1 terminator construct (pCVAC117). The psaD terminator not only worked well in combination with psaDP, but also with B2-TubulinP (pCVAC167). The psaD terminator in combination with B2-TubulinP increased luciferase expression by 10 fold when compared to B2-TubulinP-Luc-B2-Tubulin terminator construct (pCVAC168). However, inclusion of an additional copy of psaD terminator did not improve luciferase expression.
In fact it significantly reduced luciferase expression when compared to single copy of \textit{psaD} terminator.

The results indicate that the \textit{psaD} terminator is perhaps more efficient at producing properly processed transcripts compared to \textit{CCP1} and \textit{B2-Tubulin} terminators and thereby increases the steady state levels of transgene transcripts resulting in enhanced transgene expression. This was confirmed by real time PCR. Clones carrying single copies of constructs pCVAC117 (\textit{psaDP-LUC-CCP1 ter}) and pCVAC157 (\textit{psaDP-LUC-psaD ter}) were identified by Southern blot (Figures 2.6 & 2.7). There was no correlation between the level of luciferase expression and the number of copies of luciferase carried by these clones. Two single copy clones from CC424/pCVAC117 (clones 18 and 25) and CC424/pCVAC157 (clones 48 and 84) were selected for carrying out real time PCR to compare transcript levels of luciferase. The transcript level of luciferase among pCVAC157 clones were found to be about 2 to 10 fold higher than luciferase transcript levels among pCVAC117 clones (Figure 2.8). Highest luciferase transcript levels were seen in CC424/pCVAC157 clone 84, where luciferase transcript levels were 10-fold higher than CC424/pCVAC117 clone 25 and almost 20-fold higher than CC424/pCVAC117 clone 18. This indicates that higher luciferase expression among clones carrying \textit{psaD} terminator is mostly due to increase in the steady state levels of luciferase transcripts. Level of luciferase expression among single copy clones of pCVAC117 and pCVAC157 was then checked by western blot (Figure 2.9). The results of western blot support the results of RLU and real time PCR experiments as highest luciferase expression was seen among CC424/pCVAC157 clone 84, followed by
CC424/pCVAC157 clone 48. CC424/pCVAC117 clones showed significantly lower luciferase expression when compared to CC424/pCVAC157 clones.
Figure 2.4: Schematic drawings of different terminator construct.

Schematic drawings of all the constructs used to study the effect of different terminators on luciferase expression. All the vectors carry the AphVIII gene as selectable marker that confers resistance to paromomycin and is driven by Hsp70A/RbcS2 fusion promoter.
Nine clones showing highest luciferase activity from each construct were selected for carrying out RLU measurement. RLU was calculated by normalizing the luciferase activity exhibited by each culture to OD\textsubscript{750} of the culture. The data was analyzed by single factor ANOVA. While the table F value is 2.16, the calculated F value was 79.98 at 2.55 \times 10^{-28} level of significance indicating that there are significant differences among the clones expressing luciferase with different terminator regions. The calculated critical difference (CD) value was 135155. Accept for the psaDP-LUC-B2-Tubulin terminator and psaDP-LUC-CCP1 terminator constructs that did not differ significantly from each other, all other constructs differed significantly from each other with respect to levels of luciferase expression. N=72

Figure 2.5: Comparison of different terminator constructs by measuring Relative luminescence Unit (RLU) activity.
Figure 2.6: Southern blot analysis of selected CC424/pCVAC117 clones.

Southern blot analysis of selected CC424/pCVAC117 (psaD-LUC-CCP1 ter) clones was carried out to identify single copy events. Total genomic DNA (5.0µg) from each clone was used for analysis. Blot was probed with Gaussia luciferase probe.
Southern blot analysis of selected CC424/pCVAC157 (psaDP-LUC-psaD ter) clones was carried out to identify single copy events. Total genomic DNA (5.0µg) from each clone was used for analysis. Blot was probed with Gaussia luciferase probe.

Figure 2.7: Southern blot analysis of selected CC424/pCVAC157 clones.
Figure 2.8: Real time PCR analysis of selected CC424/pCVAC117 and CC424/pCVAC157 clones.

CC424/pCVAC117 (psaDP-LUC-CCP1 ter) clones 18 and 25, and CC424/pCVAC157 (psaDP-LUC-psaD ter) clones 48 and 84, carrying single copy of luciferase were analyzed by real time PCR to check the transcript levels of luciferase. Chlamydomonas CβLP gene was used for normalization. The mean ΔΔCT values from three experiments were subjected to analysis through single factor ANOVA. While the table F value is 3.47, the calculated F value was 77.92 at 1.7 x 10^{-7} level of significance indicating that there were significant differences among the clones expressing luciferase with different terminator regions. The calculated critical difference (CD) value was 0.49. Comparison of all the clones using CD value indicates that all the clones had significantly different levels of luciferase transcripts. The experiment was repeated three times (N=3) with four replications each time.
Figure 2.9: Western blot analysis of selected single transgene integration events from CC424/pCVAC117 (psaDP-LUC-CCP1 ter) and CC424/pCVAC157 (psaDP-LUC-psaD ter) clones.

a) Total soluble protein extract from cells equivalent to one milliliters of OD<sub>750</sub>=1 was loaded from each culture. b) Culture supernatant from one milliliters of OD<sub>750</sub>=1 was loaded from each sample. Blot was probed with anti Gaussia luciferase antibody. Dual color precision plus protein ladder was used. The two pink bands in the ladder represent bands of 25 kDa and 75 kDa in size.
2.3.3 Transformation of Chlamydomonas with expression cassette is more efficient

The percentage of clones showing luciferase activity with different promoter constructs ranged from 77.1% with pCVAC113 construct to 36.5% with pCVAC115 construct (Figure 2.10). Prior to Chlamydomonas transformation, while all the constructs except the pCVAC115 construct were restriction digested with PstI/KpnI enzymes to excise the expression cassettes carrying selection marker and luciferase together from plasmid backbone, the pCVAC115 construct was only linearized with PstI enzyme because the FeaI promoter carries a KpnI site. There appeared to be a direct correlation between the state of plasmid used for Chlamydomonas transformation and the percentage of clones showing luciferase expression. All the constructs in which the two expression cassettes were separated from the plasmid backbone showed a significantly higher percentage of clones exhibiting luciferase activity when compared to the FeaI construct. Kindle (85) also reported the effect of state of plasmid on the efficiency of transformation, however they were only looking at expression of the selection marker. Moreover they complemented a mutant strain of Chlamydomonas with the wild type gene and hence in a strict sense they were not looking at transgene expression.

We decided to evaluate the effect of state of plasmid used for Chlamydomonas transformation on transgene expression and transformation efficiency. The psaDP–LUC-CCPl terminator construct (pCVAC117) was selected for this experiment. Equal numbers of Chlamydomonas cells were transformed with 1µg of supercoiled plasmid, plasmid linearized with KpnI or PstI and plasmid digested with KpnI/PstI respectively (digested fragments were not gel purified and total 1.0 µg of digested plasmid DNA was
used for Chlamydomonas transformation). The experiment was repeated three times. Transformation efficiency was measured by counting all the paromomycin resistant colonies from each experiment. All the colonies were assayed for luciferase expression to calculate the percentage of colonies showing luciferase expression. All the data was analyzed by single factor ANOVA. With respect to the number of transformants recovered, no significant difference was found between KpnI linearized plasmid and expression cassette treatments, but these two treatments differed significantly from PstI linearized and supercoiled plasmid treatments. The PstI linearized treatment also differed significantly from the supercoiled plasmid treatment (Figure 2.11). Highest numbers of transformants were observed when the expression cassette was excised from the plasmid backbone and lowest numbers were observed with the supercoiled plasmid (Figure 2.11). The transformation efficiency with expression cassette was $2.0 \times 10^{-5}$ (207 colonies), which was 2.35 fold higher than the transformation efficiency of $8.8 \times 10^{-6}$ (88 colonies) observed with supercoiled plasmid. The state of the plasmid used for Chlamydomonas transformation also influenced the percentage of the transformants expressing luciferase significantly. The percentage of transformants showing luciferase expression ranged from 70.89% with expression cassette to 31.6% with supercoiled plasmid (Figure 2.12). While the difference between expression cassette and other treatments was significant, no significant difference was observed among either the linearized plasmid treatments or supercoiled and PstI treatments with respect to the percentage of transformants expressing luciferase. However, the KpnI treatment differed significantly from the supercoiled plasmid treatment.
The results indicate that Chlamydomonas transformation with expression cassette is more efficient in terms of number of transformants recovered and percentage of colonies showing transgene expression when compared to either linearized plasmid or supercoiled plasmid.
Figure 2.10: Percentage of colonies selected on TAP-Paromomycin plates showing luciferase expression with different promoter constructs.

Percentage of colonies selected on TAP-paromomycin plates showing luciferase expression with different promoter constructs. While constructs pCVAC113, pCVAC117, pCVAC120, pCVAC123, pCVAC129 and pCVAC130 were digested with Not1/Kpn1 restriction enzymes to separate the expression cassettes carrying luciferase and selection marker from vector backbone, construct pCVAC115 was only linearized with restriction enzyme Kpn1 prior to Chlamydomonas nuclear transformation. Eighty three clones from each construct were tested for luciferase expression.
Figure 2.11: Number of paromomycin resistant colonies obtained by transforming $1 \times 10^7$ Chlamydomonas cells/µg of supercoiled, linearized and KpnI/PstI digested pCVAC117 (psaDP-LUC-CCP1 ter) vector.

This experiment was repeated three times. The data was analyzed by single factor ANOVA. While the table F value is 4.07, the calculated F value was 9.18 at $5 \times 10^{-3}$ level of significance indicating that there were significant differences among the number of transformants obtained following transformation of equal number of Chlamydomonas cells with either supercoiled, linearized plasmid or expression cassette alone. The calculated critical difference (CD) value is 50.53. While no significant difference was found between KpnI and expression cassette treatments, these two treatments differed significantly from PstI and supercoiled plasmid treatments with respect to the number of paromomycin resistant colonies recoverd following Chlamydomonas transformation. The PstI and supercoiled plasmid treatments also differed significantly. N=3
Figure 2.12: Percentage of paromomycin resistant colonies obtained with supercoiled, linearized and KpnI/PstI digested pCVAC117 (psaDP-LUC-CCP1 ter) vector showing luciferase expression.

This experiment was repeated three times. The data was analyzed by single factor ANOVA. While the table F value is 4.07, the calculated F value was 7.62 at $1 \times 10^{-3}$ level of significance indicating that there are significant differences among the percentage of transformants exhibiting luciferase expression following transformation of equal number of Chlamydomonas cells with either supercoiled, linearized plasmid or expression cassette alone. The calculated critical difference (CD) value is 16.55. While the difference between expression cassette and other treatments was significant, no significant difference was observed among either the linearized plasmid treatments or supercoiled and PstI treatments. The KpnI treatment differed significantly from the supercoiled plasmid. N=3
2.3.4 Expression of transgenes is relatively stable in Chlamydomonas

There have been conflicting reports regarding transgene stability in Chlamydomonas. While Cerruti et al. (26) observed that about 50% of spectinomycin resistant colonies reverted to spectinomycin sensitive phenotype when grown under non selective conditions for a few generations, stable expression of *AphVIII* (hygromycin resistance) was observed by Berthold et al. (15) even after seven months of cultivation on nonselective media. Most of the studies conducted on evaluating transgene stability in Chlamydomonas have employed some kind of selection marker. A gene of interest is often introduced into Chlamydomonas along with a selection marker and the transformants are selected based on the expression of selection marker. This, however, does not ensure expression of the gene of interest. Moreover since Chlamydomonas possess the machinery for gene silencing, there is a very high probability that even though Chlamydomonas transformants continue to grow on selection media, the expression of the gene of interest may be inhibited through one of the several gene silencing mechanisms. This may explain the persistent problem of failure to detect expression of unselected transgenes from nuclear genome of Chlamydomonas. Since no proper study has been conducted to study stability of expression of transgenes other than selection markers, we decided to evaluate the stability of expression of luciferase in clones maintained on paromomycin plates. Another important reason for carrying out this experiment was due to the fact that of all the colonies selected based on paromomycin resistance with various constructs in this study, only about 30-77% of the colonies showed luciferase expression (Figure 2.10). There are potentially two possible
explanations why all paromomycin resistant clones do not show luciferase activity: (i) the clones lack the complete luciferase expression cassette, or (ii) the luciferase expression has been silenced, at least in some of the clones. It is also possible that with each passing generation the percentage of colonies showing luciferase expression may decrease significantly as observed by Cerruti et al. (26) for spectinomycin resistance.

Hence we decided to check the stability of luciferase expression among 80 Chlamydomonas transformants from four different constructs over a period of 12 months. Twenty clones exhibiting good luciferase activity were picked from each of the following constructs: pCVAC113, pCVAC115, pCVAC117 and pCVAC120. These clones were screened for luciferase activity 6 months and 12 months after the initial luciferase expression screening date. While none of the colonies from pCVAC113 and pCVAC117 constructs showed any loss of luciferase expression, one colony from pCVAC115 construct and seven colonies from pCVAC120 construct showed loss of luciferase expression at 6 month stage (Figure 2.13). At 12 month stage, one colony each from pCVAC113 and pCVAC115 and seven colonies (35%) from pCVAC120 construct showed loss of luciferase expression. No colony from pCVAC117 construct showed loss of luciferase expression after 12 months.
Figure 2.13: Number of transgenic Chlamydomonas colonies from different constructs showing luciferase silencing 6 months and 12 months after initial luciferase assay.
2.3.5 Inclusion of Nucleic Acid Binding protein1 (NAB1) site following the start codon of luciferase results in light dependent regulation of luciferase expression

In Chlamydomonas, the expression of Light Harvesting Complex (LHC) gene family, which encodes the light harvesting chlorophyll binding proteins (LHCII) of photosystemII (PSII) is regulated transcriptionally and post transcriptionally (55, 95, 110, 167). One of the modes of posttranscriptional regulation of LHCII polypeptides is through binding of Nucleic Acid Binding protein1 (NAB1) to the transcripts of LHCII genes, thereby repressing translation. This regulation is light dependent. Under conditions of sufficient light, NABI binds to LHCII transcripts and inhibits translation thereby reducing the size of the LHC antenna and protecting Chlamydomonas from photoxidative damage. Under conditions of low light, NABI does not bind to LHC transcripts allowing synthesis of LHCII polypeptides resulting in larger antenna. Binding of NAB1 to LHCII transcripts serves two functions: (i). It inhibits translation of LHCII transcripts and (ii) it stabilizes LHCII transcripts.

We explored the effect of incorporating the NAB1 binding site as part of luciferase coding sequence. The objective was to check whether inclusion of NAB1 binding site would result in light-dependent regulation of luciferase expression. The 13bp NAB1 binding site was fused to luciferase coding sequence following the start codon of luciferase by PCR and cloned downstream of psaD promoter in pSL18 vector creating plasmid pCVAC105 (psaDP-NAB1bs-LUC-psaD ter) (Figure 2.14). CC424 strain of Chlamydomonas was transformed and 83 paromomycin resistant colonies were screened for luciferase expression. Nine colonies showing highest luciferase expression were
picked to study light dependent regulation of luciferase expression. The selected colonies
were grown under low light (40 μE m\(^{-2}\)s\(^{-1}\)) and moderately high light (500 μE m\(^{-2}\)s\(^{-1}\))
conditions. Selected pCVAC157 (psaDP-LUC-psaD ter) transformants were included as
control. Luciferase activity of all the cultures was measured and normalized to OD\(_{750}\) of
the culture to calculate RLU. The data was analyzed using single factor ANOVA. The
results indicate that there was no significant effect of NAB1 binding site on light
dependent regulation of luciferase expression among pCVAC105 clones (Figure 2.15).
This result is contrary to the results obtained in our laboratory where in the inclusion of
NAB1 binding site as part of coding sequence of chlorophyll a oxygenase (CAO) resulted
in light dependent regulation of CAO expression (123).

However, the pCVAC105 clones showed a significantly lower luciferase
expression when compared to pCVAC157 clones. This indicates that either there is some
binding of NAB1 to the luciferase transcript irrespective of the light conditions resulting
in reduced expression of luciferase or the addition of six amino acids as a consequence of
fusing the NAB1 binding site to the coding sequence of luciferase following start codon
has deleterious effect on luciferase activity. To ascertain whether the reduction in
luciferase expression among pCVAC105 clones was due to N-terminus fusion of six
amino acids or due to the translational inhibition as a result of binding of NAB1 to the
NAB1 binding site, we decided to make two constructs. The first construct, pCVAC133
carries a mutant NAB1 binding site. The mutant NAB1 site was fused to the luciferase
following the start codon and codes for the same six amino acids coded by the NAB1
binding site, but differ in nucleotide composition from the NAB1 binding site. The

second construct, pCVAC163 carries the NAB1 binding site 9 nucleotide bases downstream of luciferase stop codon (Figure 2.14). We expected that the NAB1 protein would either fail to recognize the mutant NAB1 binding site or bind to it inefficiently allowing better luciferase expression when compared to pCVAC105 construct. However if the level of luciferase expression among pCVAC105 and pCVAC133 clones remains similar, then we can conclude that the addition of six amino acids at the N-terminus of luciferase as a consequence of fusing NAB1/mutantNAB1 binding sites affects luciferase activity. Similarly presence of NAB1 binding site following stop codon might allow better expression of luciferase as NAB1 binding could stabilize the luciferase transcript without inhibiting translation significantly due to the presence of the NAB1 binding site downstream of the stop codon. Moreover it also eliminates any possible effects on the activity of luciferase as a consequence of fusion of any amino acids to the luciferase protein. CC424 strain of Chlamydomonas was transformed with pCVAC133 and pCVAC163 constructs. Ninety three paromomycin resistant clones were screened for luciferase expression. Nine clones showing highest luciferase expression from constructs pCVAC105, pCVAC157, pCVAC133 and pCVAC163 were grown under low light conditions and subjected to RLU analysis. The data was analyzed using single factor ANOVA. Highest luciferase expression was seen among pCVAC157 clones, followed by pCVAC163, pCVAC133 and pCVAC105 clones in that order (Figure 2.16). Since pCVAC133 clones expressing the same protein (6aa-luciferase fusion protein) as pCVAC105 clones had significantly higher luciferase activity when compared to pCVAC105 clones, it indicates that the reduced luciferase activity among pCVAC105
clones is probably not due to fusion of six amino acids to the N-terminus of luciferase but perhaps due to inhibition of luciferase translation as a result of binding of NAB1 to the NAB1 binding site in luciferase transcript. The replacement of NAB1 binding site with mutant NAB1 binding site (in pCVAC133) possibly reduces the binding efficiency of NAB1 and thereby results in higher luciferase expression among pCVAC133 clones compared to pCVAC105 clones. However, since pCVAC163 clones carrying the NAB1 binding site downstream of the luciferase stop codon had higher luciferase activity (not significant though) when compared to pCVAC133 clones, it is probable that the NAB1 trans-acting factor is able to inhibit translation of luciferase by binding to the mutant NAB1 binding site present in the luciferase transcript of the pCVAC133 clones. The presence of significantly higher luciferase activity among pCVAC157 clones compared to pCVAC163 clones also signifies that perhaps NAB1 binding to the NAB1 binding site, present 9 nucleotide bases downstream of the luciferase stop codon among luciferase transcripts in pCVAC163 clones, can also inhibit luciferase expression. This also means that perhaps, irrespective of light conditions there is some binding of NAB1 to the NAB1 binding site. Hence even though inclusion of NAB1 results in providing some control on modulating transgene expression (123), the significant reduction in transgene expression caused by inclusion of NAB1 offsets this advantage.
Figure 2.14: Schematic diagram of constructs used to study the effect of including NAB1 binding site as part of luciferase coding sequence on luciferase expression.

All the constructs were identical in all aspects, except for the location of NAB1 binding site and the differences in the sequence of NAB1 and mutantNAB1 binding site.
Figure 2.15: NAB1 mediated light dependent regulation of luciferase expression among pCVAC105 clones.

NAB1 mediated light dependent regulation of luciferase expression among pCVAC105 (psaDP-NAB1bs-LUC-psaD ter) clones. Nine clones from each construct were grown under low light (40 µE m$^{-2}$s$^{-1}$) and moderately high light (500 µE m$^{-2}$s$^{-1}$) conditions and level of luciferase expression was determined by RLU. The data was analyzed using single factor ANOVA. Since the table F value is 4.49, while the calculated F value is 0.34 at 5 x 10$^{-2}$ level of significance, it indicates that there was no significant difference among pCVAC105 clones grown under different light conditions as far as luciferase expression is concerned. N=18
Figure 2.16: Effect of including NAB1 binding site as part of luciferase coding sequence on luciferase expression.

Nine clones showing highest luciferase activity from each construct were selected for carrying out RLU measurement. RLU was calculated by normalizing the luciferase activity exhibited by each culture to OD\textsubscript{750} of the culture. All the cultures were grown under low light (40 µE m\textsuperscript{-2} s\textsuperscript{-1}) conditions. The data was analyzed by single factor ANOVA. While the table F value is 2.62, the calculated F value is 14.38 at 3.09 x 10\textsuperscript{-7} level of significance indicating that the various NAB1 binding site-luciferase constructs differ significantly as far as luciferase expression is concerned. The calculated critical difference (CD) value is 231888. Comparison of clones using CD value indicates that while constructs pCVAC133 differs significantly from construct pCVAC105, it does not differ significantly from pCVAC163 with respect to levels of luciferase expression. Construct pCVAC157 had significantly higher luciferase expression compared to any other construct. N=45
2.3.6  uvm4 and uvm11 strains of Chlamydomonas do not offer any advantage for transgene expression

The uvm4 and uvm11 strains are two mutant strains of Chlamydomonas that were developed by UV mutagenesis (114). The authors have proposed that these strains allow good expression of transgenes with reduced transgene silencing. Since our objective was to achieve highest possible expression of transgenes, we decided to compare these strains with CC424 strain by checking level of luciferase expression. The uvm4, uvm11 and CC424 strains were transformed with pCVAC117 construct. 83 paromomycin resistant colonies from each strain were screened for luciferase expression. Six colonies showing highest expression from each strain were picked for comparing luciferase expression. Luciferase activity was measured and normalized to OD$_{750}$ of the culture to calculate RLU. The data was analyzed by single factor ANOVA. Significantly high luciferase expression was observed with CC424 strain compared to uvm11 strain. The difference in the level of luciferase expression between CC424 and uvm4 or uvm4 and uvm11 was not significant (Figure 2.17). The results indicate that uvm4 and uvm11 strains offer no advantage over CC424 strain as far as luciferase expression is concerned.
Figure 2.17: Comparision of luciferase expression levels among selected CC424/pCVAC117, uvm4/ pCVAC117 and uvm11/ pCVAC117 clones.

Five clones showing highest luciferase activity from each construct were selected for carrying out RLU measurement. RLU was calculated by normalizing the luciferase activity exhibited by each culture to OD$_{750}$ of the culture. The results indicate that uvm strains offer no advantage as far as luciferase expression is concerned. The data was analyzed by single factor ANOVA. While the table F value is 3.68, the calculated F value was 5.027 at 2% level of significance indicating that there are significant differences among the three strains with respect to luciferase expression. The calculated critical difference (CD) value is 19894. Comparision of strains using the CD value indicates that while strain CC424/pCVAC117 show significantly higher luciferase expression compared to uvm11/pCVAC117 clones, they do not differ significantly from uvm4/pCVAC117. There is no significant difference between uvm4/pCVAC117 and uvm11/pCVAC117 clones. N=18
2.4 Discussion

2.4.1 Not only promoter, but terminator also has significant role in transgene expression

Even though the first report on nuclear transformation of Chlamydomonas was published more than 20 years ago, the problem of poor levels of transgene expression from the nuclear genome of Chlamydomonas has not been solved. In spite of having several advantages over other biological protein production systems such as low cost of production, short multiplication time, ability to carry out post translational modification of proteins and ability to direct secretion of proteins into the culture medium permitting easy and cost effective purification, the problem of low levels of transgene expression has prevented Chlamydomonas from becoming the preferred system for production of recombinant proteins. In this study we have tried to address some of the fundamental questions concerning transgene expression from nuclear genome of Chlamydomonas.

Several heterologous and Chlamydomonas promoters have been used for transgene expression in Chlamydomonas including B2-Tubulin (33), cabII-1 (17), Rbcs2 (59), Hsp70A, Hsp70A/Rbcs2 fusion (143), CopI (59), psaD (54), CaMV35S and variants of CaMV35S (138, 159). Several studies have compared some of these promoters, however, no comprehensive comparison of these promoters has been done till date. Luciferase from Gaussia princeps was employed as reporter to compare strength of several widely used Chlamydomonas promoters and two heterologous viral promoters. Constructs identical in all aspect except for the promoter driving the reporter gene were used to transform Chlamydomonas and promoter strength was determined by measuring
RLU. The widely used Chlamydomonas promoter, *psaD* was found to be the best promoter for transgene expression in Chlamydomonas. Luciferase expression from *psaD* promoter was about 85 fold higher than the weakest promoter, *Fea1*. The Chlamydomonas *Actin* and the widely used *Hsp70A/Rbcs2* fusion promoters came second and third respectively. Among constitutive promoters, lowest expression was found with the *B2-Tubulin* promoter. Surprisingly the heterologous viral promoters performed poorly. Several heterologous viral promoters, including *CaMV35S* are widely used for transgene expression in plants. However there are significant differences between the genome composition of plants and Chlamydomonas which can have significant impact on the ability of these viral promoters to drive gene expression in Chlamydomonas. The GC content of Chlamydomonas genome is high, about 64% (107), while that of *CaMV35S* and *CVDP* promoters is 46.35 and 42.3 percent respectively. Therefore the Chlamydomonas transcriptional machinery probably does not efficiently initiate transcription from the cis-regulatory elements found among viral promoters leading to poor expression of transgenes driven by these promoters.

The *psaD* gene codes for the alpha subunit of photosystem I. The coding sequence of *psaD* lacks any introns and probably this contributes towards its ability to drive excellent expression from transgenes lacking introns (54). The Chlamydomonas *Actin* promoter used in this study performed well and is a good addition to the repertoire of promoters available for transgene expression in Chlamydomonas to researchers. Nevertheless we observed that the impact of transcription terminator on transgene expression was much stronger than promoter.
Even though the terminator plays a very important role in gene expression, not much work has been done to compare and identify an efficient terminator for transgene expression in either plants or Chlamydomonas. We compared the effect of terminator regions of *CCP1*, *B2-Tubulin* and *psaD* on luciferase expression. These three terminators were used in combination with *B2-Tubulin* and *psaD* promoters. Significantly high luciferase expression was observed among clones carrying *psaD* terminator. The average luciferase expression level in clones with *psaD* terminator was 6 to 10 fold higher than clones carrying luciferase expression driven by the same promoter but flanked by a different terminator. Since the terminator regions are implicated in regulating gene expression by controlling transcription termination and 3’ end processing of mRNA, it’s possible that the *psaD* terminator is more efficient at carrying out these processes when compared to *B2-Tubulin* or *CCP1* terminators, resulting in higher luciferase expression. Similar observations with different terminators have also been made in plants (76, 111). While Ingelbrecht et al. (76) observed a 60 fold difference in the level of GUS expression in tobacco plants harboring terminator of *RBCS2* and chalcone synthase, Nagaya et al. (111) observed a two fold increase in GUS expression in Arabidopsis plants with terminator from *Hsp18.2* when compared to *NOS* terminator. Luo and Chen (99) found that while use of *CaMV35S* terminator resulted in higher GUS expression when compared to *NOS* terminator, inclusion of both of these terminators together (*CaMV35S* and *NOS* terminators together) further increased the GUS expression.

The terminators are responsible for transcript termination and 3’ end processing. Proper 3’ end processing of transcripts plays a very important role in gene expression as
it determines the stability of transcript, nuclear to cytoplasmic transport and translation competence of the transcript (178). In all the studies with GUS reporter in plants, the increased GUS expression showed direct co-relation with increase in the level of GUS transcripts. This indicates that an efficient terminator enhances gene expression by improving the stability of transcripts. Luo and Chen (99) also found that transformation of a GUS expressing Arabidopsis line with a GUS construct lacking terminator leads to GUS silencing. They showed that lack of terminator leads to production of aberrant transcripts that are not properly processed leading to GUS silencing. Similarly use of weak terminator signals also results in lower transgene expression as it allows read through transcription leading to improper transcript termination and processing, reducing transcript stability and promoting gene silencing leading to reduced transgene expression. That is why use of CaMV35S and NOS terminators together increased GUS expression by 3 to 4 folds when compared to single terminator (99). Luciferase transcript levels were evaluated among single copy transformants of pCVAC117 (psaDP-LUC-CCP1 ter) and pCVAC157 (psaDP-LUC-psaD ter) by real time PCR. The pCVAC157 clones had 2 to 10-fold higher luciferase transcripts when compared to pCVAC117 transformants. This indicates that the psaD terminator increases luciferase expression by increasing the steady state levels of luciferase transcript perhaps through more efficient processing of the transcripts or enhanced stabilization.

Moreover the psaD terminator was not only effective in improving transgene expression in combination with psaD promoter. When used in combination with B2-TubulinP, the psaD terminator increased luciferase expression by 10-fold when compared
to the $B2$-$TubulinP$-$LUC$-$B2$-$Tubulin$ ter construct. This indicates that the $psaD$ terminator can be used in combination with other promoters to improve transgene expression. The possibility of further improving luciferase expression by employing two copies of $psaD$ terminator was also explored. However, use of two copies of $psaD$ terminator appeared to be counterproductive as it resulted in significant reduction in luciferase expression when compared to single copy of $psaD$ terminator. This shows that employing two copies of terminator is effective in improving transgene expression only in case of weak terminator signals that allow transcript read through and this strategy does not work with strong and efficient terminator signals.

The results point to the fact that transgene expression in Chlamydomonas can be significantly improved by employing more efficient terminators and facilitate towards realizing the true potential of Chlamydomonas for molecular farming.

2.4.2 Efficiency of Chlamydomonas transformation is influenced by the state of the plasmid used for transformation.

We observed a direct correlation between the state of the plasmid used for Chlamydomonas transformation and transformation efficiency and the percentage of clones showing transgene expression. The transformation efficiency observed with expression cassette and linearized plasmid was about 2 fold higher than that with the supercoiled plasmid. The percentage of clones showing luciferase expression increased from 32% with supercoiled plasmid to 77% with expression cassettes (Selection marker and gene of interest together). The number was 53% with linearized plasmid. Increase in Chlamydomonas transformation efficiency by using linearized plasmid over supercoiled
plasmid was first reported by Kindle (85) and since then it has become a norm to use linearized plasmid for Chlamydomonas transformation. The exact reason how transformation with the linearized plasmid/expression cassette leads to increase in transformation efficiency and the number of clones showing transgene expression has not been determined. Most probably the use of linearized plasmid /expression cassette facilitates integration of transgene into the genomic DNA of Chlamydomonas over supercoiled plasmid resulting in better transformation efficiency. Linear DNA may also integrate preferentially in transcriptionally active regions with double stranded breaks by end-joining repair mechanisms resulting in better transformation efficiency.

The Chlamydomonas genome is GC rich (65%), while the plasmids being derived from bacteria are AT rich. Transformation of Chlamydomonas with linearized/supercoiled plasmid leads to integration of plasmid backbone into the Chlamydomonas genome. Chlamydomonas cellular machinery probably recognizes the AT rich plasmid sequences as alien and silences them and the surrounding regions including the gene of interest introduced into Chlamydomonas leading to transgene silencing and reducing the number of transformants expressing transgene. Transformation of Chlamydomonas with expression cassettes does not suffer from this draw back and thus results in a significantly higher percentage of transformants expressing gene of interest.

2.4.3 Stability of transgene expression in Chlamydomonas in not a major problem

The stability of transgene expression in Chlamydomonas has been evaluated using either antibiotic resistance selection markers (15, 26) or by complementing a mutant strain of Chlamydomonas with the wild-type gene (17, 85). Studies with antibiotic
selection markers have shown that while transgene expression in Chlamydomonas remains more or less stable when the transgenic colonies are maintained under selection pressure, expression of transgenes is lost within few generations of removing selection pressure (26). Most of the time a gene of interest is introduced along with the selection marker in Chlamydomonas to produce the desired phenotype or to overexpress the protein of interest. While the colonies are selected for and maintained using the selection agent, there is no way to select for transgenic Chlamydomonas clones that express the transgene of interest in most cases. Hence transgenic colonies may shut down the expression of gene of interest while continue to survive by expressing selection marker.

Achieving desired levels of transgene expression in Chlamydomonas has been a problem, and since Chlamydomonas is known to posses the machinery required for gene silencing, it’s most likely that the transgene expression is inhibited by employing one or the other form of gene silencing (TGS or PTGS). Therefore we decided to evaluate the stability of expression of luciferase in clones maintained on paromomycin plates. Totally 80 clones from four different constructs were evaluated for stability of luciferase expression over 12 month period. While none of the clone from construct pCVAC117 showed any loss of luciferase expression, one clone (5%) each from pCVAC113 and pCVAC115 and 7 clones (35%) from pCVAC120 constructs showed loss of luciferase expression after 12 months.

The results indicate that transgene silencing in Chlamydomonas appear to be mostly established during initial stages/ generations of a transgenic colony and the clones that are not affected by transgene silencing during initial stages remain relatively stable.
and continue to maintain good expression levels of the transgene. The significantly high frequency of clones showing luciferase silencing among pCVAC120 construct is most likely due to the fact that in this construct the expression of luciferase as well as the selection marker (*AphVIII*) is driven by the same promoter, *Hsp70A/Rbcs2* which probably leads to homology dependent transcriptional gene silencing (104, 119). Studies with plants (103, 119) have also shown that presence of more than one copy of a single promoter driving transgene expression leads to homology dependent transcriptional gene silencing. Homology of as small as 90bp region among two promoters can lead homology dependent gene silencing (119). Consequently it is recommended that expression of selection marker and the gene of interest should be driven by different promoters. Overall the results indicate that stability of transgene expression in Chlamydomonas does not appear to be major concern provided that the gene of interest and the selection marker are driven by different promoters.

2.4.4 Inclusion of Nucleic Acid Binding protein1 (NAB1) site following the start codon of luciferase and the uvm strains do not offer any advantage for transgene expression

The NAB1 protein binds to the transcripts of the light harvesting chlorophyll binding proteins in a light dependent fashion and modulates the size of the LHCII antenna complex (110) in Chlamydomonas. The binding of NAB1 to the 13bp long NAB1 binding site found in the transcripts of LHCII polypeptides inhibits translation but stabilizes the transcripts (110, 167). This regulation helps Chlamydomonas
photosynthetic machinery to deal with fluctuating light conditions and prevent photooxidative damage. This NAB1 mediated light dependent regulation of LHCII antenna size has been employed to increase the photosynthetic efficiency of Chlamydomonas. Transformation of a NAB1 knockout strain of Chlamydomonas with a mutant form of NAB1 protein that is permanently active leads to 10 to 17% reduction in the LHC antenna size and 50% increase in photosynthetic efficiency (14). We employed this mechanism to check if luciferase expression can be regulated by light. The NAB1 binding site was fused to the coding sequence of luciferase following the start codon. Transformants carrying NAB1-LUC (pCVAC105) construct grown under moderately high light conditions showed about 9% reduction in luciferase expression when compared to cultures grown under low light conditions (Figure 2.13) indicating that there is some light dependent regulation of luciferase expression. However, the luciferase expression among the pCVAC105 transformants was significantly lower than the control construct pCVAC157, indicating that the inclusion of NAB1 binding site perhaps leads to severe translational repression. This implies that the NAB1 mediated gene regulation mechanism cannot be employed to enhance transgene expression.

The uvm4 and uvm11 strains developed through UV mutagenesis (114) were claimed to allow higher expression of transgenes. We compared these strains with the control CC424 strain by checking luciferase expression. Nine clones from each construct were tested. The results show that the uvm strains do not offer any specific advantage over CC424 strain as far as luciferase expression is concerned. Infact luciferase expression among uvm clones was significantly lower than CC424.
CHAPTER 3: EXPRESSION OF HUMAN PARAOXONASE1 (PON1) AND AHL LACTONASE FROM BACILLUS SPS IN CHLAMYDOMONAS REINHARDTII

3.1 Introduction

The demand for recombinant proteins for therapeutic and industrial applications is ever increasing. This demand will continue to grow with the discovery and engineering of new proteins with diverse functions. Currently, bacteria, yeast, insect and mammalian cell cultures, and plants are used for production of recombinant proteins (44). While the production of recombinant proteins from insect and mammalian cell lines is expensive, the costs involved in eliminating endotoxins from proteins produced using bacterial systems for therapeutic purposes makes these systems unattractive. Moreover, bacterial and even yeast systems to some extent lack the machinery required for post-translational modification of proteins such as glycosylation that often contributes to the function and stability of many eukaryotic proteins (20). Even though plants are an attractive alternative to these systems, several issues related to protein purification and low yields need to be resolved (164). Hence there is need to develop new recombinant protein production systems that do not suffer from the drawbacks listed above.
We propose developing the micro-alga *Chlamydomonas reinhardtii* as a system for production of recombinant proteins. *Chlamydomonas* was selected because 1) it is considered as a safe food-grade material 2) it is non-toxic and non-pathogenic, 3) it is a model genetic organism which can be easily transformed 4) it can be grown on minimal medium thereby reducing cost of production 5) being a eukaryote, it has the machinery for post translational modification of proteins 6) availability of mutant strains that allow secretion of the protein into the medium facilitating protein purification and 7) stable transgenic lines can be generated in less than 3 weeks and production can be scaled up in few months (106). The present work demonstrates that *Chlamydomonas* can be used as a recombinant protein production system with the expression of two proteins of pharmaceutical value from two diverse sources. We have expressed human paraoxonase1 (*PON1*) and AHL lactonase from *Bacillus sps* and shown that these proteins were functional.

*PON1* has several pharmaceutical applications. It is synthesized in the liver and transported along with high density lipoproteins (HDL) in the plasma and functions as an antioxidant preventing the oxidation of low density lipoproteins (LDL) and thereby atherosclerosis (150). It can also hydrolyze organophosphate (OP) (nerve gases) substrates into biologically inert products without getting consumed in the process of detoxifying the nerve agent (90, 94). *PON1* can be used to treat OP pesticide and nerve gas toxicity as rabbit or human *PON1* purified from serum has demonstrated therapeutic potential for treating OP exposures in mice both before and after OP exposure (156).
However, the highly hydrophobic nature of \textit{PON1} makes its expression in various cell systems very challenging. Till date, \textit{PON1} has only been expressed in \textit{E.coli} (3).

The AHL lactonase (AiiA) enzyme produced by various \textit{Bacillus sps}, disrupts the quorum sensing (QS) among gram negative bacteria by destroying the QS signal molecules N-acyl homoserine lactones (AHL) (41, 43). It acts by hydrolyzing the homoserine lactone ring of AHLs. The potential pharmaceutical value of AHL lactonase was demonstrated when fish injected with AHL lactonase (28) and plants expressing AHL lactonase were shown to be resistant to bacterial infections (41). It has also been demonstrated that Chlamydomonas makes molecular mimics of the AHL class of QS molecules of unknown structure (128). Thus, expression of lactonase in Chlamydomonas will also help us determine if the algal mimic compounds are susceptible to inactivation by lactonase. In this work we have demonstrated successful expression of the \textit{PON1} and AHL lactonase proteins in Chlamydomonas.

3.2 Materials and Methods

3.2.1 Algal strains and cultural conditions

Chlamydomonas strains CC424 (cw15, arg2, sr-u-2-60 mt\textsuperscript{-}) and CC741 (ac-u-(beta) mt\textsuperscript{+}) were obtained from the Chlamydomonas culture collection at Duke University, USA. Strains UVM4 and UVM11 were kindly provided by Dr. R. Bock (Max Planck institute, Germany). Strains were grown mixotrophically in liquid or on solid TAP medium (69) at 23\degree C under continuous white light (40 \text{ mE m}^{-2}\text{s}^{-1}), unless otherwise stated. Medium was supplemented with 100 \text{ \mu g/mL} of arginine when required. Selection of nuclear transformants was performed by using solid TAP medium or TAP medium
supplemented with 100 µg/mL of arginine and 50 µg/mL of paromomycin or 10 µg/mL of hygromycin. Selection of chloroplast transformants using strain CC741 was performed with high salt (HS) medium.

### 3.2.2 Vector Construction

Human *PON1* (genebank accession AF320003) was codon optimized for Chlamydomonas nuclear expression using Graphical Codon Usage Analyser 2.0 (www.gcua.de). The codon optimized version was synthesized by Epoch Biolabs, Inc (Sugar Land, TX, USA). *Gaussia princeps* luciferase lacking stop codon was cloned into Ndel/EcoRI sites of vector pSL18 (39) creating plasmid pCVAC158. Codon optimized *PON1* was then amplified and cloned into EcoRI/XbaI sites of pCVAC158 fusing *PON1* to C-terminus of luciferase, creating plasmid pLP. Codon optimized *PON1* lacking stop codon was also cloned into Ndel/EcoRI sites of vector pCVAC122 fusing *PON1* to N-terminus of luciferase, creating vector pPL. Codon optimized *PON1* was also cloned into Ndel/XbaI sites of pSL18 creating plasmid pPON. G2E6 (2) was also cloned into Ndel/XbaI sites of pSL18 creating plasmid pG2E6. The G2E6 polar (*huPON1* with surface polar mutation from G2E6 and G3C9 (2) were cloned in the chloroplast transformation vector pBA155 (G2E6p was kindly provided by Dr. Thomas Magliery, OSU, Columbus, OH, USA) creating plasmids pCG2E6p and pCG3C9. All the primers used for cloning the above mentioned *PON1* variants are listed in Table 3.1.

Vector construction for AHL lactonase was done as described by Rajamani (128). Briefly, codon optimized aiiA for Chlamydomons nuclear expression assembled using the oligo shuffling technique described by Stemmer et al. (154) was kindly provided by
Dr. Max Teplitski (University of Florida, Gainesville, FL, USA). The codon optimized gene lacking stop codon was PCR amplified with primers aiiAFwd (5’-CGGGCATATGGTCGACACCGTGAAAGCTGTACTTCG-3’) and aiiARev (5’-GGCCGGGCGCTCCTCCCGAGATGTATTCGGGGAACACCTTG-3’), introducing Nde I and Not I sites at 5’ and 3’ ends respectively. Codon optimized CFP gene for Chlamydomonas nuclear expression was amplified using primers, CFPFwd (5’-CCCGGGCGGCCGCAAGGGCGAGGAGCTGTTCACCG-3’) and CFPRev (5’-CCTTTGCGGCGCCCTTACTTGTACAGCTCGTCCATGCCGTGGG-3’), introducing Not I and Nar I restriction sites respectively. The PCR amplified aiiA and CFP were sequentially cloned into plasmid pSSCR7 (147), creating pSSCR7-EuaimC.

AHL lactonase–YFP fusion was cloned in bacterial expression vector pQE-30 (Qiagen, Valencia, CA, USA) for overexpression. The AHL lactonase (aiiA) coding sequence was amplified with primers aiiAFwd (5’-CCGGTACCATGACAGTAAAGAAGCTTTATTTCGTCCCAGC-3’) and aiiARev (5’-CGCGGCGCCGCGATATATATTCCAGGAACACTTTACATCCC-3’), restriction digested with KpnI/NotI and cloned into same sites of vector pQE30-CLPY (128) creating plasmid pQE30-AimY containing AHL lactonase-YFP fusion.
Table 3.1: List of primers used to construct various vectors with PON variants

<table>
<thead>
<tr>
<th>VECTOR</th>
<th>GENE</th>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>RESTRICTION SITE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSL18</td>
<td>PON1</td>
<td>FPON1</td>
<td>5’-GAA ATC ATA TGA TGG GCA AGC TGA TCG CGC TGA CCC T-3’</td>
<td>NdeI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPON1</td>
<td>5’-ATC TTT CTA GAT TAC AGC TCG CAG TAC AGG GCC TT-3’</td>
<td>XbaI</td>
</tr>
<tr>
<td></td>
<td>G2E6</td>
<td>FG2E6</td>
<td>5’-CAG ACA TAT GGC TAA GCT GAC AGC GCT CAC-3’</td>
<td>NdeI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RG2E6</td>
<td>5’-AGC ATC TAG ATT ACA GAT CAC AGT AAA GAG CTT TGT GAA ACA-3’</td>
<td>XbaI</td>
</tr>
<tr>
<td>PCVAC158</td>
<td>LUC</td>
<td>fFPON</td>
<td>5’-ATC TTG AAT TCA TGG CGA AGC TGA TCG CGC TGA CCC T-3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPON1</td>
<td>SAME USED TO CLONE PON1</td>
<td>XbaI</td>
</tr>
<tr>
<td>PCVAC122</td>
<td>PON</td>
<td>FPON1</td>
<td>SAME USED TO CLONE PON1</td>
<td>NdeI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPON1f</td>
<td>5’-TGT CAG AAT TCC AGC TCG CAG TAC AGG GCC TT-3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nostop</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHLOROPLAST pBA155</td>
<td>G3C9</td>
<td>FG3C9c</td>
<td>5’-TAT ACC ATG GCT AAA CTG ACA GCG CTC ACA CTC TTG-3’</td>
<td>NcoI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RG3C9c</td>
<td>5’-TAT AGC ATG CTT ATT ACA GCT CAC AGT AAA GA-3’</td>
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</tr>
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<td></td>
<td></td>
<td>polar</td>
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</tr>
<tr>
<td></td>
<td>G2E6</td>
<td>FG2E6pc</td>
<td>5’-TAT ACC ATG GCA AAG CTG ACC GCG CTG ACT CTG TTA G-3’</td>
<td>NcoI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RG2E6pc</td>
<td>5’-CTG TGC ATG CTT AGC CGC TAC TTC CTA ATT CAC AG-3’</td>
<td>SphI</td>
</tr>
</tbody>
</table>
3.2.3 Nuclear transformation of *C. reinhardtii*

*Chlamydomonas reinhardtii* nuclear transformation was performed using the glass beads method (85). Briefly, CC424 strain of Chlamydomonas was grown in 100 mL of TAP liquid media supplemented with arginine. Cells were harvested in log phase (OD$_{750}$=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 4 mL of sterile TAP+40 µM sucrose. Resuspended cells (300 µL) were transferred to a sterile microcentrifuge tube containing 300 mg of sterile glass beads. (0.425-0.6 mm, Sigma, USA) 100 µL of sterile 20% PEG 6000 (Sigma, USA) was added to the cells along with 1.5 µg of plasmid DNA. Prior to transformation, all the constructs were restriction digested either to linearize the construct or to excise the two expression cassettes carrying selection marker and gene of interest together, from the plasmid backbone. Following addition of plasmid DNA, cells were vortexed for 20 seconds and plated on to TAP agar plates containing 100 µg/mL arginine and 50 µg/mL paromomycin or 10 µg/mL hygromycin and 100 µg/mL arginine.

For plasmids lacking any selection marker (pSSCR7 backbone), co-transformation was done. For co-transformation, CC424 strain was transformed using glass beads method following addition of the linearized target plasmid (3 µg DNA) and the plasmid harboring the Arg7 gene, p389 (1 µg DNA). Cells were plated on TAP agar plates without arginine.

3.2.4 *Chlamydomonas* Chloroplast transformation

Chlamydomonas chloroplast transformation was performed following the protocol described by Ishikura et al. (77). Briefly, psbA deletion strain (CC741) of
Chlamydomonas was grown in 100 mL of TAP liquid media. Cells were harvested in log phase (OD\textsubscript{750}=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 2 mL of sterile HS medium. About 300µL of cells were spread in the center of HS agar plates. Gold particles (1µm) (InBio Gold, Eltham, Victoria, Australia) coated with plasmid DNAs were shot into Chlamydomonas cells on the agar plate using a Bio-Rad PDS 1000 He Biolistic gun (Bio-Rad, Hercules, CA, USA) at 1100 psi under vacuum. Following shooting, cells were plated onto HS agar plates for selection.

3.2.5 PCR analysis

PCR analysis was performed as described by Rajamani (128). Genomic DNA was extracted from putative transformants growing on selection medium using a modified xanthine mini prep method described by Dr. Steve Surzycki (http://www.chlamy.org/methods/dna.html). A half loop of algal cells were resuspended in 300 µL of xanthogenate buffer (12.5 mM potassium ethyl xanthogenate, 100 mM Tris-HCl pH 7.5, 80 mM EDTA pH 8.5, 700 mM NaCl) and incubated at 65\°C water for 1.0 hour. Following incubation, the cell suspension was centrifuged for 10 minutes (14,000 rpm) to collect the supernatant. The supernatant was transferred to a fresh microcentrifuge tube and 2.5 volume of cold 95% ethanol (750 µL) was added. The solution was mixed well by inverting the tube several times allowing DNA to precipitate. The samples were then centrifuged for 5 min (14,000 rpm) to pellet the DNA. The DNA pellet was washed with 700 µL of cold 70% ethanol and centrifuged for 3.0 min. The ethanol was removed by decanting and the DNA pellet was dried using a Speedvac to get rid of any residual ethanol. The DNA pellet was then resuspended in 100µL of sterile
double distilled water and 2-5 µL of the DNA sample was used as template for setting PCR.

3.2.6 Luciferase assay

Luciferase assays were performed at room temperature with POLARstar OPTIMA microplate luminometer (BMG Labtech, Germany) with automated substrate injection. For screening Chlamydomonas transformants for luciferase expression, transformants were inoculated in 2 mL 96 well (Nunc, Rochester, NY, USA) plates containing TAP media and grown for 2 to 3 days under continuous light. One hundred microliters of algal culture was diluted with 100 µL of TAP medium in 96 well plate and luminescence was recorded after automatic injection of 10 µL coelenterazine (0.1 mM in 10% ethanol) (NanoLight technologies, Pinetop, AZ USA) every 4 seconds for total of 24 seconds at the highest amplification and directly referred as luminescent units. Secretion of luciferase into the culture medium was confirmed by measuring luciferase activity in culture supernatant. One hundred micro liters of culture supernatant obtained following centrifugation at 12,000 × g was mixed with 100 µL of liquid TAP medium in a 96 well plate for measuring luminescence.

3.2.7 Paraoxonase Activity

Paraoxonase activity was measured with paraoxon (O,O-diethyl-O-4-nitrophenylphosphate) (60) and with O-ethyl methylphosphonyl (EMP) (8). Paraoxonase activity was measured using 100 µL of cells from culture with OD_{750}=1.0,. Cells were collected by centrifugation and resuspended in 100 µL of reaction buffer (50 mM Tris-HCl, 10 mM CaCl₂, at pH 7.4) with or without 0.1% Tergitol. Cells were then lysed by
freeze thaw (3x). The whole mix of lysed cells in buffer or the supernatant after centrifuging the debris were used to measure the esterase activity. The reaction was carried out in 96 well plates with 100 µL of sample, 4 µL of 0.13M paraoxon or 7 µL 0.5 mM EMP and reaction volume was made up to 200µL with Reaction Buffer. The reaction was monitored for 30 min at 412 nm for paraoxon or at 405nm for 10 min with EMP using SpecraMax M5 (Molecular Devices, Sunnyvale, CA, USA). The product formation was calculated using the molar absorptivity (paraoxon $\varepsilon=10515$ M$^{-1}$ cm$^{-1}$ and EMP $\varepsilon=37000$ M$^{-1}$ cm$^{-1}$) following the Beer-Lambert law, divided by the total soluble protein in the cell lysate estimated by Bradford assay.

3.2.8 Immunoblot analysis

Cells equivalent to one milliliters of OD$_{750}$=1 from algal cultures were pelleted in microcentrifuge tubes by centrifugation at 10,000 rpm for 1 minute at room temperature and resuspended in 50 µl of 1X Laemmli loading dye (0.063M Tris-HCl Ph 6.8, 2% SDS-PAGE, 10% glycerol, 5% B-mercaptaethanol, 0.05% bromo-phenol-blue), and incubated at 100°C for 5 minutes. Samples were removed from water bath, cooled to room temperature, centrifuged at 10,000 rpm for 2 minutes and the cell extract was separated on 10% SDS polyacrylamide gel by electrophoresis. Protein samples were transferred to PVDF membrane (Millipore, Bllerica, MA, USA) by semi-dry blotting. Membrane carrying luciferase-PON1 fusion samples was incubated with anti G-LUC rabbit polyclonal antibody (Nanolight technologies, USA), at 1: 1000 dilution at 4°C for overnight following blocking of membrane with 0.5% BSA in TBS containing 0.05% Tween-20. After washing the blot for 3 x 5minutes with TBST (TBS +0.05% Tween
20), blot was incubated with anti-rabbit IgG antibody (Sigma, Saint Louis, MO, USA) conjugated to horse-radish peroxidase at 1:10000 dilution for 2 hours at room temperature. Following three washes with TBST, detection was completed by chemiluminescence using luminol (0.4 mM) and iodophenol (8.0 mM) (Sigma, Saint Louis, MO, USA (173).

Membrane carrying AHL lactonase-CFP fusion was incubated with anti-GFP rabbit polyclonal antibody (Invitrogen, Carlsbad, CA, USA), at 1: 1000 dilution at 4°C for overnight following blocking of membrane with 0.5% BSA in TBS containing 0.05% Tween-20. After washing the blot for 3 x 5minutes with TBST (TBS +0.05% Tween 20), blot was incubated with anti-rabbit IgG antibody (Sigma, Saint Louis, MO, USA) conjugated to horse-radish peroxidase at 1:10000 dilution for 2 hours at room temperature. Following three washes with TBST, detection was completed by chemiluminescence using luminol and iodophenol (Sigma, Saint Louis, MO, USA) (173).

### 3.2.9 AHL mimic purification from *C. reinhardtii*

AHL mimics produced by Chlamydomonas were purified following the protocol described by Rajamani (128). Log phase cultures of Chlamydomonas (OD_{750}=0.8 to 1.0) were used to inoculate 1.0 liter of HS medium at 5% inoculum and grown under continuous light at 24°C for 12 to 16 days. Algal culture filtrate obtained following centrifugation (10,000 g for 10 min at 20°C) and filtration through 0.8 µm nitrocellulose membranes were extracted twice with 0.3 volumes of ethyl acetate. The extracts were rotary evaporated at 35 to 36°C to dryness and resuspended in 600 µL of 50% acetonitrile:water (v/v). The extracts were fractionated on a reverse-phase C18 (RP-C18)
analytical HPLC column (5µm, 250-4LiChrospher100, Agilent Technologies, Palo Alto, CA, USA) mounted with C18 guard column. The injected sample (200 µL) was eluted at a flow rate of 0.75 mL/min from HPLC column for 5 min with water, then for 40 min with a linear gradient of increasing acetonitrile from 0% to 100% (v/v), followed by elution with 100% acetonitrile for an additional 15 min. HPLC fractions of 750 µL were collected and 100 µL samples of each fraction were dried and analyzed for CepR activity using Pseudomonas putida F117 (pAS-C8) reporter.

3.2.10 AHL lactonase-YFP purification

AHL lactonase-YFP fusion protein was purified following the protocol described by Rajamani (128). BL21 cells carrying pQE-AimY were grown at 28°C in LB broth supplemented with 100 µg/mL ampicillin until the cell density reaches OD₆₀₀=0.6. Protein expression was then induced with 0.3 mM isopropyl thiogalactoside and cells were grown for additional 6 hours. Cells were harvested by centrifugation and cell pellet was resuspended in buffer A (25 mM NaH₂PO₄-Na₂HPO₄ (pH 7.0), 50 mM NaCl, and 10 mM imidazole) and sonicated with 15 mM 2-mercaptoethanol and 1.0 mM phenylmethyl sulfonyl fluoride. Following centrifugation, the clear cell lysate supernatant was loaded onto a His-Select™-HC nickel affinity gel (Sigma) column equilibrated with buffer A. The column was washed with several column volumes buffer A and protein was eluted with 25 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl, and 150 mM imidazole. Protein purity was determined by running samples on SDS/PAGE. Protein concentration was determined and 50µL aliquots of protein (0.6 mg/mL) were made and stored at -80°C.
3.2.11 Preparation of CepR reporter strain P. putida for bioassay

The CepR reporter strain (153) was prepared for bioassays following the protocol of Rajamani (128). The strain was grown in half-strength LB supplemented with gentamicin (25 µg/mL) at 30ºC for overnight. The overnight grown culture was diluted 1 to 4 times in fresh half LB and grown for 1 hour. For bioassay, 100 µL of the reporter was added to the 96 well micro titer plate containing air-dried HPLC fractions. GFP fluorescence from the plate was recorded 3 hours after incubation with the reporter strain using a Victor multiwall plate reader.

3.2.12 AHL lactonase assay with C8-HSL and CepR reporter mimic activities

AHL lactonase-YFP fusion protein activity was assayed as described by Rajamani (128). Briefly, 80 µL of C8-HSL at concentrations of 4 µM, 40 nM and 400 nM in 10 mM sodium phosphate buffer pH 7.0 were transferred to 96-well microtiter plate. AHL lactonase-YFP fusion protein (2.5 µL) was then added to each well and the plates were incubated at 30ºC for 6 hours. The plates were then removed, air-dried overnight and bioassay was carried out with P. putida reporter strain. For mimic inactivation assay, the HPLC fractions were first air-dried in a 96-well microtiter plate and resuspended in 80 µL of 10 mM sodium phosphate buffer pH 7.0. Then the procedure described for C8-HSL inactivation assay was followed. Proper blank (80 µL of buffer) and positive (400 nM of C8-HSL) controls were included in all the experiments.

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3.3 Results

3.3.1 Expression of different PON1 variants in Chlamydomonas

This work was done in collaboration with Dr. Vanessa Falcao (Post doctoral associate, Sayre lab, Ohio State University, Columbus, Ohio). I was involved in cloning of PON1 and PON1 variants in Chlamydomonas nuclear and chloroplast transformation vectors and performed nuclear and chloroplast transformation of Chlamydomonas and Dr. Falcao analyzed the transformants for PON1 expression. Codon optimized humanPON1 for Chlamydomonas nuclear expression and the more soluble variant of rabbitPON1 G2E6 (3) were cloned into nuclear transformation vector pSL18 (Figure 3.1). uvm4 strain (114) of Chlamydomonas was transformed with these constructs and transgenic colonies were recovered by plating the cells on TAP medium supplemented with paromomycin. uvm4 strain was used because it has been reported to allow high expression of transgenes (114). All the clones were screened for paraoxonase activity. Twenty four percent of PON1 (17/72) and 9% of G2E6 (8/86) clones showed paraoxonase activity. Presence of PON1/G2E6 coding sequence in colonies showing paraoxonase activity was confirmed by PCR (Figure 3.2). PCR positive clones showing paraoxonase activity were subjected to immunoblot analysis using anti PON antibody. However, no band corresponding to PON1 or G2E6 was detected in immunoblot experiments. Since these proteins are highly hydrophobic, the proteins perhaps remained in the insoluble fraction resulting in failure of immunoblot experiment. This was confirmed by repeating the paraoxonase activity assay on the insoluble and soluble fractions of cell lysate from colonies that were positive for paraoxonase activity. Most of
the activity was observed in the insoluble fraction while little or no activity was observed from the soluble fraction of cell lysate. Hence, reverse transcriptase (RT)-PCR reaction was set to test for presence of PON1 transcripts. Transcripts of right size were detected in the PCR positive clones indicating that these clones were transcribing the transgenes (Figure 3.2). Results of RT-PCR experiment coupled with the paraoxonase activity assay indicate that these clones were producing the PON1 protein and the protein was functional. The transgenic clones are stable and have survived for more than two years. The recovery of stable Chlamydomonas transformants expressing PON1/G2E6 and their ability to survive for more than 2 years indicates that Chlamydomonas provides an alternative to PON1 expression in E.coli, which to our knowledge is the only alternative platform for expressing this protein.

To facilitate solubilization, screening, proper folding and expression of PON1, we fused Gaussia luciferase to the N and C terminus region of PON1 (Figure 3.1). Since the Gaussia luciferase is known to be secreted into the media by the cell wall mutant strains of Chlamydomonas, we expected that the signal peptide of luciferase would perhaps guide the passage of the fusion protein through the secretory pathway resulting in glycosylation of PON1. The colonies obtained after transformation were screened for luciferase activity. While only 2% (2/48) of the colonies expressing luciferase–PON1 fusion protein showed luciferase activity, 21% (10/46) of the colonies expressing PON1-luciferase fusion protein were positive for luciferase activity. These selected colonies were then tested for paraoxonase activity. While good paraoxonase activity was observed among PON1-luciferase clones (Figure 3.6), very low paraoxonase activity was observed
among luciferase-\textit{PON1} clones. Protein extract from \textit{PON1}-luciferase clones was treated with 0.1% tergitol to improve solubilization of proteins for immunoblot analysis. This treatment appeared to be effective in solubilizing \textit{PON1}-luciferase fusion protein as a 65kDa band representing the fusion protein was observed among the clone (Figure 3.3). The immunoblot results also indicate that perhaps fusion of \textit{PON1} with luciferase improved solubility of \textit{PON1} as we were able to see the 65 kDa \textit{PON1}-luciferase band and all of the fusion protein was not lost in the insoluble fraction as observed when \textit{PON1} was expressed alone.
Figure 3.1: Schematic representation of *PON1/G2E6* constructs for Chlamydomonas nuclear expression

Schematic representation of *PON1/G2E6* constructs for Chlamydomonas nuclear expression. All the constructs were identical in all aspects except for the gene of interest. Expression of gene of interest was driven by psaD promoter. All the constructs were restriction digested with Not1/Kpn1 restriction enzymes to separate the vector backbone from the two expression cassettes before Chlamydomonas transformation. All the vectors carry the AphVIII gene as selectable marker that confers resistance to paromomycin and is driven by Hsp70A/Rbsc2 hybrid promoter.
Figure 3.2: PCR and RT-PCR analysis of CC424/pPON clones

A) PCR analysis of CC424/pPON clones. B) RT-PCR analysis of CC424/pPON clones. Transcript of expected size was seen in all the clones. Empty vector (EV), Water control (-), Plasmid control (+). This figure was contributed by Dr. Vanessa Falcao.
Expression of PON-luciferase fusion protein in selected UVM4/pPL clone showing good paraoxonase activity was tested by immunoblot analysis. Blot was probed with anti PON antibodies. Arrows indicate the PON-luciferase fusion band in the transgenic clone and the 20kDa luciferase band in the control. Chlamydomonas transformed with empty vector (EV) and Chlamydomonas expressing luciferase (LUC) were included as controls. Dual color precision plus protein ladder was used. This figure was contributed by Dr. Vanessa Falcao.
3.3.2 Expression of PON1 variants in Chlamydomonas Chloroplast

For chloroplast expression, more soluble variants of rabbit PON1, G2E6 and G3C9 (3) were used. G2E6 and G3C9 genes were developed by random mutagenesis of rabbit PON1 and selected for increased solubility and activity against paraoxon. The G2E6 and G3C9 genes were cloned into chloroplast transformation vector pBA155 (Figure 3.4). The psbA deletion strain of Chlamydomonas, CC4147 was transformed with these constructs by particle bombardment. Transgenic colonies were selected based on their ability to carry out photosynthesis and grow on HS plates. Selected colonies were tested for paraoxonase activity with paroxon. The level of paraoxonase activity observed among G2E6 chloroplast transformants was similar to the activity observed among G2E6 nuclear and transformants (Figure 3.6). Again the paraoxonase activity was observed in the insoluble fraction of the cell lysate. To solubilize proteins for immunoblot analysis, several treatments were tried. Best results were obtained with 0.1% tergitol as we were able to observe a band corresponding to G3C9 (50 kDa) in western blot (Figure 3.5). In addition to the 50 kDa band, a 60 kDa band was also seen in the blot. This 60 kDa band probably is due to the lipophilic characteristic of paraoxonases, resulting from the association of G3C9 with some lipid.
Figure 3.4: Schematic representation of G2E6 and G3C9 constructs for Chlamydomonas chloroplast expression.

Schematic representation of G2E6 and G3C9 constructs for Chlamydomonas chloroplast expression. Expression of gene of interest was driven by atpA promoter. Expression cassette was flanked by psbA sequences for integration into psbA site. The psbA gene also served as the selection marker.
Figure 3.5: Expression of PON variant G3C9 in Chlamydomonas chloroplast.

Immunoblot analysis of selected CC4147/pCG3C9 clone showing good paraoxonase activity. Blot was probed with anti PON antibody. Two bands of 50 kDa and 60 kDa were seen in the clone. These bands were absent in the empty vector control. The 50 kDa corresponds to the normal size of PON1. EV: Empty vector control. Dual color precision plus protein ladder was used. This figure was contributed by Dr. Vanessa Falcao.
3.3.3 *PON1* and *PON1* variants expressed in Chlamydomonas were functional

This work was done by Dr. Vanessa Falcao. The nuclear and chloroplast transformants of Chlamydomonas expressing *PON1* and its variants were screened for paraoxonase activity using two different substrates, paraoxon and EMP. It was observed that independent of the variant being expressed by a clone, most of the protein remained associated with the insoluble fraction of cell lysate and no effective activity was observed in the soluble phase. The different *PON1* variants show different affinities for different substrates (60). We found G2E6 expressed by Chlamydomonas nuclear and chloroplast transformants to be most active with paraoxon while *PON1*-luciferase fusion expressed from nuclear genome to be most active with EMP (Figure 3.6). The level of activity shown by the nuclear expressed *PON1* (pPON) and chloroplast expressed G2E6 (pCG2E6) and G3C9 (pCG3C9) relative to *E.coli* expressed G2E6 were calculated (Table 3.2). The activity of *E.coli* expressed, purified G2E6 was provided by Dr. Thomas Magilery (OSU, Columbus, OH, USA). The chloroplast expressed G2E6 showed highest paraoxonase activity followed by the nuclear expressed *PON1*.
Figure 3.6: Analysis of Paraoxonase activity of PON1 and its variants expressed in Chlamydomonas.

Paraoxonase activity of PON1 and its variants expressed in Chlamydomonas was measured with A) 2.6 mM of paraoxon as substrate by monitoring absorbance at OD\textsubscript{412} B) or 17.5 µM of O-ethyl methylphosphonyl (EMP) as substrate by monitoring absorbance at OD\textsubscript{405}. Activities were measured using 100 µl of cell lysate from cultures harvested at an OD\textsubscript{750} = 1. Cells were resuspended in buffer (50mM Tris Hcl, 10mM CaCl2 pH 7.4) and lysed by freeze-thaw method. The product formation was calculated using the molar absorptivity (Paraoxon \( \varepsilon = 10515 \text{ M}^{-1} \text{ cm}^{-1} \) and EMP \( \varepsilon = 37000 \text{ M}^{-1} \text{ cm}^{-1} \)) following the Beer-Lambert law, divided by the total soluble protein in solution estimated by Bradford assay. G2E6 and MBP-PON1 expressed and purified from \textit{E. coli} were used as controls in Fig. A and B respectively. This figure was contributed by Dr. Vanessa Falcao.
Table 3.2: Relative expression levels of \textit{PON1} and \textit{PON1} variants in Chlamydomonas using paraoxon as substrate based on the specific activity of G2E6 expressed in \textit{E. coli}.

This table was contributed by Dr. Vanessa Falcao.

<table>
<thead>
<tr>
<th>Constructs with best activity</th>
<th>nM of product/min/ug of total protein</th>
<th>Levels of expression of \textit{PON1}/variants in Chlamy calculated as percentage of µg of total soluble protein extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPON</td>
<td>7</td>
<td>0.2%</td>
</tr>
<tr>
<td>pG3C9 chloroplast</td>
<td>4</td>
<td>0.1%</td>
</tr>
<tr>
<td>pG2E6 chloroplast</td>
<td>10</td>
<td>0.3%</td>
</tr>
</tbody>
</table>
3.3.4 AHL signal mimics produced by *C. reinhardtii* are inactivated by AHL lactonase

This work was done in collaboration with Dr. Sathish Rajamani (former graduate student, Sayre lab, Ohio State University, Columbus, Ohio). Dr. Rajamani did all the work involved in developing transgenic Chlamydomonas expressing AHL lactonase and overexpression and purification of AHL lactonase enzyme from *E.coli*, while I did only the two experiments described below. The unicellular microalga Chlamydomonas is known to secrete compounds that mimic the activity of the N-acyl-L-homoserine lactone (AHL) signal molecules that activate the CepR and LasR AHL bacterial quorum sensing reporter strains (161). The AHL lactonase enzyme, produced by various *Bacillus* sps., inactivates the bacterial AHL signals by hydrolyzing the homoserine lactone ring of AHL’s (42). Moreover the AHL signal mimics produced by plants are also inactivated by the AHL lactonase (37, 62). To test whether the signal mimics produced by Chlamydomonas share some characteristics with the AHL’s such as conserved lactone ring, the Chlamydomonas CepR mimic fractions were treated with AHL lactonase. Significant reduction in the mimic activities of the selected fractions was observed following AHL lactonase treatment (Figure 3.7) indicating that these mimic compounds and AHLs perhaps have lactone ring in common. It also indicates that Chlamydomonas perhaps employs these mimic compounds to inhibit bacterial populations in its natural habitat.
Figure 3.7: In vitro inactivation of CepR mimics of Chlamydomonas by AHL lactonase.

Dried HPLC fractions from 12-day-old, cell-wall defective *C. reinhardtii* (CC-424) cultures were treated with active (open circles) or heat-inactivated AiiA enzyme (filled squares) and bioassayed with the *P. putida* F117 (pAS-C8)-CepR reporter. The percentage stimulation was calculated by comparing the reporter response to the fully induced reporter using C8-HSL.
3.3.5 AHL lactonase-CFP fusion protein produced in Chlamydomonas is functional

Since AHL lactonase is a protein of pharmaceutical value, we decided to express this protein in Chlamydomonas. The objective was to test whether the protein produced in Chlamydomonas is functional and what effect it has on the production of AHL mimics by Chlamydomonas. AHL lactonase was expressed as fusion protein with CFP in Chlamydomonas. Before making the AHL lactonase-CFP fusion, we had confirmed functionality of AHL lactonase-YFP purified from bacteria (128). CC424 strain of Chlamydomonas was transformed with pSSCR7-EuaimC construct. Colonies growing on selection medium were screened by PCR to confirm presence of AHL lactonase coding sequence. A band of ~1.5kb was seen in a number of clones (Figure 3.8). These selected clones were subjected to immunoblot analysis to confirm expression of AHL lactonase-CFP fusion protein using anti GFP antibody. However, large numbers of nonspecific bands were seen in the blot and it was difficult to conclude whether AHL lactonase-CFP protein was produced by these PCR positive clones. Since our objective was to test whether the protein produced in Chlamydomonas is functional and whether it effects the production of AHL mimics produced by Chlamydomonas, we decided to purify the mimics from selected transgenics and test their activities. AHL mimics were purified from transgenic lines EuaimC 1, 26 and 72 along with CC424 and bioassay with CepR reporter was carried out to test the profile of mimic compounds. The transgenics showed 60 to 80% and 40 to 60% reduction in CepR stimulating activities of fraction 33 and 30 respectively when compared to non transgenic control (CC424), significant reduction in
CepR stimulating activities of other fractions from transgenics was also observed (Figure 3.9), indicating that AHL lactonase-CFP fusion perhaps is interfering with the AHL mimics production in transgenic clones. There was drastic reduction in the CepR stimulating ability of fractions 29 and 32 among transgenic lines. This data strongly suggests that the AHL lactonase-CFP fusion protein produced in Chlamydomonas is functional, however, structural identification of the Chlamydomonas mimic compounds is required before we are fully confident of this interpretation.
Figure 3.8: PCR analysis of CC424/AHL lactonase-CFP clones.

PCR confirmation of CC424/AHL lactonase-CFP clones (lane 2 to 10). Plasmid control (lane 11) and CC424 (lane 12) were included as positive and negative controls respectively. This figure was contributed by Dr. Sathish Rajamani.
Figure 3.9: CepR mimic activities of transgenic algae expressing AHL lactonase-CFP fusion.

Twelve days old cultures of *Chlamydomonas* CC-424 and AHL lactonase-CFP transgenics (AimC-1, -26, -72) were HPLC fractionated and assayed for CepR mimic activity production using the *P. putida* F117 (pAS-C8)-CepR reporter strain. The percentage stimulation was calculated by comparing the reporter response to the fully induced reporter using C8-HSL.
3.4 Discussion

In this work we have shown that Chlamydomonas can be used as a system for expression of recombinant proteins. Two proteins of pharmaceutical value, human *PON1* and bacterial AHL lactonase were expressed in Chlamydomonas and were shown to be functional.

3.4.1 Expression of functional human *PON1* and its variants in Chlamydomonas

*PON1* has several therapeutic properties. *PON1* is a highly glycosylated protein and in serum it remains associated with HDL. This makes expression of *PON1* in bacterial and other expression system problematic as *PON1* in the absence of HDL is highly hydrophobic and becomes toxic to host cells. There has been some success with bacterial expression of slightly less hydrophobic mutants of rabbit *PON1*, G2E6(2), however still very little protein is obtained in the soluble phase. To date the only way to produce paraoxonase for therapeutical applications is through purification from mammals, which makes its production very expensive. Hence the possibility of expressing *PON1* in Chlamydomonas was explored since several human proteins such as human erythropoietin (48), single chain antibody (105) and human glutamic acid decarboxylase 65 (164) have been successfully expressed in Chlamydomonas.

Expression of *PON1* and its variants was observed in Chlamydomonas nuclear as well as chloroplast transformants. The proteins expressed in Chlamydomonas appeared to be insoluble since the paraoxonase activity was observed in the insoluble fraction of cell lysate. This was expected because *PON1* remains associated with HDL through Apoliprotein A1 (ApoA1) in blood serum (160). In the absence of ApoA1, *PON1*
becomes highly hydrophobic and toxic to host cells. However, even though PON1 and its variants were insoluble, they were not toxic to Chlamydomonas cells as transgenics have survived for more than 2 years now. This is a very promising result because till date no transgenic plant expressing PON1 has been recovered due to toxicity associated with expression of PON1. Fusion of luciferase to C-terminus of PON1 (PON1-luciferase) appeared to improve the solubility of the protein as the fusion protein was detected in immunoblot experiments. However, Chlamydomonas clones expressing luciferase fused to the hydrophobic N-terminus of PON1 (luciferase-PON1) showed low paraoxonase activity, indicating that probably the N-terminus luciferase fusion interferes with proper folding of PON1 and disrupts its structure resulting in low paraoxonase activity. The highly hydrophobic nature of N-terminus perhaps affects folding of luciferase also because very few clones with pLP construct (2%) showed luciferase activity.

Chlamydomonas chloroplast transformants also showed good paraoxonase activity, reconfirming the finding that PON1 activity is independent of glycosylation and probably glycosylation only contributes towards stability of protein (45). The different PON1 variants have different affinities for the different substrates (60). In our experiments, G2E6 polar expressed in the chloroplast was most active with paraoxon while PON1-luciferase fusion was most active with EMP. However, it is difficult to directly compare the activities of PON1 and its variants expressed in Chlamydomonas as we did not use purified proteins for the assays. The differences in the activities with different substrates might be due to different levels of the proteins in the samples. Hence, much more needs to be done before we can say whether the nuclear expression is better
than chloroplast expression or vice versa. One of the important experiments that needs to be done is expression of *PON1* along with ApoA1 in Chlamydomonas to see whether this improves the solubility and activity of *PON1*.

### 3.4.2 Expression of functional AHL lactonase in Chlamydomonas

With increase in bacterial resistance to antibiotics, new strategies to control bacterial infections are being explored. One of these strategies involves controlling bacterial infections by interfering with the quorum sensing (QS) mechanism found in bacteria (127). AHL lactonase found in various *Bacillus sps* inactivates AHLs, the quorum sensing signal molecules and interfere with QS. In fact plants expressing AHL lactonase were found to be resistant to bacterial infection (41), while plants expressing proteins that promote synthesis of AHLs were found to be hyper-susceptible to bacterial infection (57, 162). Similarly, compounds that mimic the bacterial QS signals also interfere with QS. Halogenated furanones produced by the marine alga *Delisea pulchra* act as AHL mimics and bind to AHL receptor polypeptides, causing receptor misfolding resulting in its proteolytic degradation, thus inhibiting QS in bacteria (63, 100). Halogenated furanones were shown to disrupt QS in *P aeruginosa* and reduce lung infections in mouse (169, 71). Since Chlamydomonas is known to secrete CepR mimics (161), we made an attempt to find out whether these mimics have any structural similarity to AHLs by subjecting the mimics to AHL lactonase treatment. AHL lactonase treatment significantly reduced the activity of mimics produced by Chlamydomonas, indicating that these mimics perhaps have a lactone ring in common with AHLs. This also means that the mimics produced by Chlamydomonas can have potential
pharmaceutical value as demonstrated for the halogenated furanones produced by *Delisea pulchra*. Transgenic Chlamydomonas expressing AHL lactonase-CFP fusion showed a significantly lower mimic activity when compared to controls, indicating that the AHL lactonase produced by these transgenics was functional and was perhaps responsible for the reduced levels of mimics in these transgenics. More experiments need to be done to study the effect of reduction in production of AHL mimics by transgenic Chlamydomonas expressing AHL lactonase-CFP on algal growth, their sensitivity to bacterial contamination or interactions with bacteria in their natural habitat.

Overall we have shown that Chlamydomonas can be used to produce difficult eukaryotic proteins such as human *PON1*. Human *PON1* and AHL lactonase produced in Chlamydomonas were functional and the QS mimic compounds produced by Chlamydomonas perhaps share lactone ring with AHLs. More experiments need to be done to optimize expression of human *PON1* in Chlamydomonas and to study the role of QS mimics produced by Chlamydomonas.
CHAPTER 4: DEVELOPMENT OF MICROALGAL ORAL VACCINES AGAINST INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS OF SALMON AND TROUT

4.1 Introduction

Infectious hematopoietic necrosis virus (IHNV) is the most important rhabdoviral pathogen of salmonids in North America infecting wild and cultured Pacific (Oncorhynchus sps.) and Atlantic salmon (Salmo salar L.) (163). The virus causes an acute disease associated with rapid viral replication in kidneys resulting in destruction of hematopoietic tissues leading to onset of mortality in 4 to 7 days post infection (124). The virus was originally considered to be limited to pacific north-west regions of America, but has now spread to other parts of the world through shipment of contaminated fish and eggs. IHNV is transmitted through contact with infected survivors and contaminated feed and the viral infection is temperature dependent (92). The disease is most severe at 10°C and rare at 15°C and above (92). Losses due to disease are usually close to 100% because the control measures require harvesting and destruction of the entire stock and decontamination of the farm or hatchery (92, 124). In spite of being such a serious threat
to aquaculture industry, no licensed vaccine is available against IHNV in United States, while a DNA vaccine has been approved for commercial use in Canada.

IHNV is a negative-sense RNA virus belonging to genus Novirhabdovirus. Its genome codes for six proteins. Of the six IHNV proteins, only IHNV glycoprotein (G) is known to induce protective immunity (31, 50). Fish are effectively protected from viral infection when vaccinated with G protein either through intramuscular injection or immersion vaccination (50). A DNA vaccine carrying G coding sequence on a plasmid under the control of the immediate-early enhancer-promoter sequences of human cytomegalovirus (CMV) was also found to be very effective in protecting fish against IHNV (6) and has been approved for commercial use in Canada. The DNA vaccine induces a strong protective immune response in fish since IHNV neutralizing antibodies are observed 6 weeks after vaccination (6, 32). The DNA vaccine has also reduced the cost of vaccination as large scale production and purification of plasmid is very cost effective compared to production and purification of G protein. Still vaccination of fish through injection of DNA vaccine is a labor intensive and tedious job and it is not practically possible to vaccinate each and every fish in a farm. Various studies have explored alternative routes of administering DNA vaccines, such as immersion and ultrasound with DNA-coated microspheres and DNA encapsulated in polysaccharide coated beads or liposomes, however none of these methods have provided protection comparable to intramuscular injection (30, 51, 52, 134, 135). To overcome problems associated with delivering vaccines to fish and to further reduce the costs associated with
antigen production, purification and antigen packaging, we propose use of the microalga *Chlamydomonas reinhardtii* as a system to deliver vaccines to fish orally.

*Chlamydomonas* was selected because it is amenable to genetic manipulation. In fact, foreign genes and proteins, including antigens can be expressed either from chloroplast, mitochondrial, or the nuclear genome. In addition to this, *Chlamydomonas* has many other characteristics that make it an excellent candidate for an oral vaccine delivery system. These include: 1) being a microalgae, it is part of the aquatic food chain, 2) and hence, it is innocuous, non-toxic and non-pathogenic, 3) both fresh water and marine species of *Chlamydomonas* can be used 4) availability of mutants that can survive and mate only under controlled conditions. The latter is very important because it addresses the biosafety concerns associated with the use of live algae to deliver vaccines to fish and the possibility of their escape into the environment. In fact to make the system more robust, some of the mutations can be stacked. In addition to these advantages, it has been shown that Chlamydomonas expressing p57 protein from *Renibacterium salmoninarum* were able to successfully induce strong immune response in fish, when vaccinated by either oral or immersion means (140, 146).

Two strategies for developing microalgal oral vaccine against IHNV were tried. The first strategy was an epitope based strategy that involved identification of G protein epitopes using phage display library and over expression of these epitopes as fusion protein with p57 protein of *Renibacterium salmoninarum* in Chlamydomonas chloroplast. Phage display was used for identification of epitopes because it produces a peptide of specific length with all possible combinations of amino acids (20^{12} peptides for a 12 mer
library) and thus can represent all possible linear epitopes that can be recognized by an antibody. p57 was selected because it is known to induce strong immune response in fish (80, 168). The second strategy involved expression of full length G protein from Chlamydomonas nuclear or chloroplast genome. While no specific immune response was observed with the p57-G epitope fusions, anti-G antibodies were detected in the immune serum of rabbits vaccinated orally with the transgenic Chlamydomonas.

4.2 Materials and Methods

4.2.1 IHNV-G epitope identification using phage display library

Putative epitopes of G protein were identified using a dodecapeptide (12 mer) phage display library (NEB, Ipswich, MA, USA). Screening of phage display library was performed using the ultralink protein G column (Pierce, Rockford, IL, USA). All the solutions were allowed to pass through the column by gravity flow. The protein G column was first equilibrated with 5 gel-bed volumes (10 mL) of TBS (20 mM Tris-HCl, 150 mM NaCl). The column was then incubated with 100 µL of mouse IgG anti rainbow trout IgM antibodies (Microtek International Inc, Saanichton, BC, Canada) diluted in 2 mL of TBS. Following rinsing of column with 6 mL of TBS, the column was filled with 5 mg/mL BSA solution in TBS and incubated for 30 minutes to block all unbound G protein surfaces. Column was again rinsed with 6 mL of TBS. The pre-immune serum from trout was diluted with TBS to have a final dilution of 1:300 in total volume of 2.0 mL. 10 µL of original phage library was added to the TBS-pre-immune sera solution, mixed well and incubated overnight at 4°C. Following overnight incubation, the TBS-
pre-immune sera-phage display library mixture was added to the column. The eluate was collected and passed through the column three times. On fourth pass of the mixture, the eluate was collected. This eluate constitutes the pre-immune subtracted library. This screening with pre-immune sera was done to get rid of phage sequences that can bind specifically and non-specifically to the antibodies present in pre-immune sera. The column was rinsed with 10 ml of TBS and the eluate was collected and mixed with the pre-immune subtracted library and stored at 4° C for overnight. The pre-immune subtracted phage library was then screened against immune serum using a new protein G column. The steps mentioned above were followed with the exception that instead of using pre-immune serum, immune serum was diluted with pre-immune sera screened library in 1:300 ratio and incubated at 4° C for overnight. The immune-serum-pre-immune serum screened library mixture was added to the column. The eluate was collected and passed through the column three times. The column was then washed with TBST (TBS + 0.1% [v/v] Tween-20) till the phage titer was less than 1.0/µL. The IgM bound phages were then eluted selectively with 6 to 10 ml of 0.2M glycine HCl (pH 2.5 to 3.0) and immediately neutralized to pH 7.5 using 1.0M Tris (pH 9.1). These phages mimic the putative G protein epitopes and were titered for plaque collection.

4.2.2 Phage titering

Phage titering was done following the manufacturer’s (NEB, Ipswich, MA, USA) protocol. Briefly, ER2738 strain of *E. coli* was grown to OD<sub>600</sub>~0.5 and 200 µL of cells were dispensed into four sterile micro-centrifuge tubes. Ten fold serial dilutions (4X) of the phages that mimic G protein epitopes were made and 10 µL of each dilution was
added to each of the four tubes containing ER2738 cells, vortexed and incubated at room temperature for 1 to 5 minutes. Phage infected cells were then transferred to a sterile tube containing 3 mL of melted agarose (incubated at 45°C), vortexed and immediately poured onto pre-warmed (37°C) LB/IPTG/Xgal plate and spread evenly by tilting. Plates were allowed to cool at room temperature for 15 minutes and then incubated at 37°C for overnight. The phage infected colonies or plaques appear blue in color.

4.2.3 Phage amplification and Sequencing

Phages were amplified and sequenced following the manufacturer’s protocol (NEB, Ipswich, MA, USA) protocol. An overnight grown culture of ER2738 was diluted in 1:100 in LB. 1.0 mL of the diluted culture was transferred to sterile culture tubes. With a sterile wooden stick, blue plaques from the tittering plate were transferred to sterile culture tubes containing diluted ER2738 culture and incubated in 37°C incubator shaker for 5 hours. The cultures were then transferred to micro-centrifuge tubes and spun at 14,000 rpm for 30 seconds. Supernatant (500 µL) was transferred to fresh micro-centrifuge tubes and 200 µL of 20% PEG/2.5M NaCl was added and tubes were inverted 8 to 10 times to mix, let stand for 10-20 minutes at room temperature. Tubes were then centrifuged at 14,000 rpm for 10 minutes at 4°C and supernatant was completely removed with a pipette. The phage pellet was completely resuspended in 100 µL of Iodide buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 4 M sodium iodide (NaI)) by tapping the tube. 250 µL of ethanol was added to tubes, mixed and tubes were incubated at room temperature for 20 minutes. The tubes were then centrifuged at 14,000 rpm for 10 minutes at 4°C, supernatant was discarded and the pellet was washed in 500 µL of
70% ethanol. Dried DNA pellet was then resuspended in 30 µL of TE buffer. DNA concentration was measured and phage DNA was sent for sequencing with -96 gIII sequencing primer (5’- CCCTCATAGTTAGCGTAACG-3’) supplied with the kit.

### 4.2.4 Algal strains and cultural conditions

Chlamydomonas strains CC424 (cw15, arg2, sr-u-2-60 mt-) and CC741 (ac-u-(beta) mt+) were obtained from the Chlamydomonas culture collection at Duke University, USA. Strains were grown mixotrophically in liquid or on solid TAP Medium (69) at 23°C under continuous white light (40 µE m⁻² s⁻¹), unless otherwise stated. Medium was supplemented with 100 µg/mL of arginine when required. Selection of nuclear transformants was performed by using solid TAP medium or TAP medium supplemented with 50 µg/mL of paromomycin and 100 µg/mL of arginine. Selection of chloroplast transformants using strain CC741 (ac-u-(beta) mt+) was performed with high salt (HS) medium.

### 4.2.5 Codon optimization of genes for Chlamydomonas nuclear and chloroplast expression.

Coding sequence of IHNV glyco protein (G) was codon optimized to achieve better expression from Chlamydomonas nuclear and chloroplast genomes. A *Chlamydomonas reinhardtii* codon usage table for nuclear genes was obtained from codon usage database (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055). Only the best codons (codons occurring at a frequency of about 10 codons or more/1000 codons in the table) were selected for each amino acid and a codon usage table was made (Table 4.1). The codons from the G coding
sequence were replaced with the preferred codons for Chlamydomonas nuclear expression manually. Forty two nucleotide base long overlapping primers were designed for assembling the codon optimized G coding sequence for Chlamydomonas nuclear expression.

The codon usage table for *Chlamydomonas reinhardtii* chloroplast genes was obtained from the codon usage database ([http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.chloroplast](http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3055.chloroplast)) and all the steps described above for codon optimization of G coding sequence for Chlamydomonas nuclear expression were followed for optimizing G coding sequence for Chlamydomonas chloroplast expression.

Codon optimized G coding sequence for Chlamydomonas nuclear and chloroplast expression were assembled following the Oligo shuffling technique of Stemmer et.al. (154)
Table 4.1: Codon usage table for codon optimizing IHNV glyco protein coding sequence for Chlamydomonas nuclear and chloroplast expression.

List of codons used to codon optimize the G coding sequence for Chlamydomonas nuclear and chloroplast expression. Where two are more codon have been used for a single amino acid, the ratio in which these codons were used is shown in parenthesis.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Preferred codons for Nuclear expression</th>
<th>Preferred codons for Chloroplast expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>ATC</td>
<td>ATT</td>
</tr>
<tr>
<td>Leucine</td>
<td>CTG, CTC (5:1)</td>
<td>TTA, CTT (7:1)</td>
</tr>
<tr>
<td>Valine</td>
<td>GTG, GTC (3:1)</td>
<td>GTT, GTA (1:1)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>TTC</td>
<td>TTT, TTC (2:1)</td>
</tr>
<tr>
<td>Methionine</td>
<td>ATG</td>
<td>ATG</td>
</tr>
<tr>
<td>Cysteine</td>
<td>TGC</td>
<td>TGT</td>
</tr>
<tr>
<td>Alanine</td>
<td>GCC, GCG, GCT (3:2:1)</td>
<td>GCT, GCA (3:2)</td>
</tr>
<tr>
<td>Glycine</td>
<td>GGC, GGT, GGG (6:1:1)</td>
<td>GGT</td>
</tr>
<tr>
<td>Proline</td>
<td>CCC, CCG (3:2)</td>
<td>CCA, CCT (3:2)</td>
</tr>
<tr>
<td>Threonine</td>
<td>ACC, ACG (2:1)</td>
<td>ACT, ACA (2:3)</td>
</tr>
<tr>
<td>Serine</td>
<td>AGC, TCG, TCC (3:2:2)</td>
<td>TCA, TCT, AGT (2:1:1)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>TAC</td>
<td>TAT, TAC (3:1)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>TGG</td>
<td>TGG</td>
</tr>
<tr>
<td>Glutamine</td>
<td>CAG</td>
<td>CAA</td>
</tr>
<tr>
<td>Asparagine</td>
<td>AAC</td>
<td>AAT, AAC (3:1)</td>
</tr>
<tr>
<td>Histidine</td>
<td>CAC</td>
<td>CAT, CAC (2:1)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>GAG</td>
<td>GAA</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>GAC</td>
<td>GAT, GAC (4:1)</td>
</tr>
<tr>
<td>Lysine</td>
<td>AAG</td>
<td>AAA</td>
</tr>
<tr>
<td>Arginine</td>
<td>CGC, CGG (3:1)</td>
<td>CGT</td>
</tr>
</tbody>
</table>
4.2.6 Vector Construction

p57 coding sequence was amplified from *Renibacterium salmoninarum* using primers p57Fwd1 (5’- TGATCATGAAAATAAAAAAAAATTTTAGCGCTGACTGC-3’) and p57Rev1 (5’- GGTGCATGCTTAGTTAAAGGTAAATATCTATTTTTTCGCTAAGGTC -3’), introducing BspHI and SphI sites at 5’ and 3’ ends, respectively. P57 coding sequence was digested with BspHI/SphI and cloned into NcoI/SphI sites of pGatpA vector (77) creating plasmid pCVAC1. The 1.7kb p57 coding sequence was excised as XhoI/SphI fragment along with a portion (about 100 bp) of atpA promoter and cloned into same sites of vector pBA155 creating plasmid pCVAC2. Selected G protein epitope coding sequences were fused to the C-terminus of p57 before the stop codon by PCR using primer atpA5’ (5’-TTAAAATTGTCGACAAAGATTTTAAAAATAAAC-3’) and overlapping primers for respective epitopes (refer table 4.1 for primer sequences). PCR products were digested with SalI/SphI and cloned into XhoI/ SphI sites of the vector pCVAC2 creating plasmids pCVAC6, pCVAC7, pCVAC8, pCVAC9, pCVAC10, pCVAC11, pCVAC12 and pCVAC13 carrying G protein epitope 1, epitope2, epitope3, epitope4, epitope5, epitope6, epitope7 and epitope8 respectively.

Codon optimized G coding sequence for Chlamydomonas nuclear and chloroplast expression were assembled following the Oligo shuffling technique of Stemmer et.al. (154). Sixty eight overlapping primers were designed for each version and coding sequence was assembled using PCR. Chloroplast codon optimized version was cloned into NcoI/SphI sites of pGATPA vector creating plasmid pCVAC20. The chloroplast
version was then taken out as XhoI/SphI fragment from pCVAC20 and cloned into same sites of pBA155 creating plasmid pCVAC21.

Nuclear version of IHNV-G was cloned into NdeI/XbaI sites of vector pSSCR7 (147) creating plasmid pCVAC24. The nuclear version was also cloned into NdeI/XbaI sites of vector pSL18 (39) creating plasmid pCVAC37. To create G- Gaussia luciferase C-terminus fusion, G lacking stop codon was amplified using primers IHNVGFwd1 (5’- ATCTTACATATGAGCGACCAGCCACCTG-3’) and IHNVGRev1 (5’- TAAGATGAATTCTGGGTGTTGCGTGTTGC-3’) and cloned into NdeI/EcoRI sites of pSL118 creating plasmid pCVAC119. Gaussia luciferase (70) was amplified with primers LUCFwd3 (5’- ATCTAGAATTCCGGGATGGGCTAGCTGAAGG-3’) and LUCRev1 (5’- AAGATAAGCTTCTAGATTACGTATGCGCCAGCCAGC-3’), introducing EcoRI and XbaI sites respectively and cloned into same sites of pCVAC119 creating vector pCVAC122. For fusing luciferase to N-terminus of G, luciferase without stop codon was amplified with primers LUCFwd1(5’- ATCTACATATGCTCGAGATGGGCGTGAAGG-3’) and LUCRev2 (5’- AAAGATGAATTCAAGCTTCTAGATTACGTATGCGCCAGCCAGC-3’), introducing sites NdeI and EcoRI respectively and cloned into NdeI/EcoRI sites of pSL18 creating plasmid pCVAC158. EcoRI and XbaI sites were then added to 5’ and 3’ of G using primers IHNVG Fwd2 (5’- AATCGGAATTCGGCGCATGAGCGCCAGCCACCACCTG-3’) and IHNVGRev (5’- CAATGCATGCGGCCGCTCTAGATTAGTGGGTGGGTTGGTGG-3’) and cloned into EcoRI/XbaI sites of pCVAC158 creating plasmid pCVAC161.
Table 4.2: List of primers used to fuse the selected eight IHNV glycoprotein putative epitopes to C-terminus of p57.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p57-Epitope1-1</td>
<td>5’-AACAGTTTTGTAATTAGCACCACATGTAATTAAGTTAA</td>
</tr>
<tr>
<td></td>
<td>AGGTAATATCTATTTTCCGC-3’</td>
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<td>p57-Epitope1-2</td>
<td>5’-GGTGCATGCTTAACCTTTCAAGTCTGTATCTGGTTGAA</td>
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<tr>
<td></td>
<td>CAGTTTGTGAATTAGCACC-3’</td>
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<tr>
<td>p57-Epitope2-1</td>
<td>5’-TTCTAAAGATGTAGCTTTTTCAACACGATTGAGTTAA</td>
</tr>
<tr>
<td></td>
<td>AGGTAATATCTATTTTCCGC-3’</td>
</tr>
<tr>
<td>p57-Epitope2-2</td>
<td>5’-GGTGCATGCTTAATGTAAACCCCATGGATGATGTTCA</td>
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<td></td>
<td>TAAGATGTAGCTTTTTACAACAC-3’</td>
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<tr>
<td>p57-Epitope3-1</td>
<td>5’-ACTTAAATAATATGGAGTAACAGAATTGTTGAGTTA</td>
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<td></td>
<td>AAGGTAATATCTATTTTCCGC-3’</td>
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<tr>
<td>p57-Epitope3-2</td>
<td>5’-GGTGCATGCTTAATGAGGTGAACGAAATTTACTTAAT</td>
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<tr>
<td></td>
<td>AAATATGGAGTAACAGAATTTG-3’</td>
</tr>
<tr>
<td>p57-Epitope4-1</td>
<td>5’-TAAAATACTAGGATGTGGAACGAAAAATTTACTAAT</td>
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<tr>
<td></td>
<td>AAGGTAATATCTATTTTCCGC-3’</td>
</tr>
<tr>
<td>p57-Epitope4-2</td>
<td>5’-GGTGCATGCTTAACATCTGTTTCTATAAAAAGCTA</td>
</tr>
<tr>
<td></td>
<td>AAATACTAGGATGTGGAACAG-3’</td>
</tr>
</tbody>
</table>

Continued
Table 4.2 (Continued)

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>p57-Epitope5-1</td>
<td>5’-TAATAATAAAAACTAATGGAATACCACCTAATGTGTA AAGGTAATATCTATTTTTTCGC-3’</td>
</tr>
<tr>
<td>p57-Epitope5-2</td>
<td>5’-GGTGCATGCTTAAATACAAAGCTGCAATAGCTAATAAT AAAACTAATGGAATACCACC-3’</td>
</tr>
<tr>
<td>p57-Epitope6-1</td>
<td>5’-TGTTAAATTAGTTGCATGATGAGCAGCATATGTGTTA AAGGTAATATCTATTTTTTCGC-3’</td>
</tr>
<tr>
<td>p57-Epitope6-2</td>
<td>5’-GGTGCATGCTTAACCAATGGACGTTCCCATTTTGGTA AAATTAGTTGCATGATGAGC-3’</td>
</tr>
<tr>
<td>p57-Epitope7-1</td>
<td>5’-ATGAGCTTTCTAACAACATTCTTCACGAGATTCTGAGTTA AAGGTAATATCTATTTTTTCGC-3’</td>
</tr>
<tr>
<td>p57-Epitope7-2</td>
<td>5’-GGTGCATGCTTAAATTGTACTAATAATTGTCATGAG CTTCTAAACATTCTTCAC-3’</td>
</tr>
<tr>
<td>p57-Epitope8-1</td>
<td>5’-TTAAATTTCTTGTGAAAAATCACATGTGTTAAAGGTA ATATCTATTTTTTCGC-3’</td>
</tr>
<tr>
<td>p57-Epitope8-2</td>
<td>5’-GGTGCATGCTTATTTTATCACCACAATAATGAGCCTTTTA ATTTCTTTGTGAAAAATCACATG-3’</td>
</tr>
</tbody>
</table>
4.2.7 Nuclear transformation of *C. reinhardtii*

*N. C. reinhardtii* nuclear transformation was performed using the glass beads method (85). Briefly, CC424 strain of Chlamydomonas was grown in 100mL of TAP liquid media supplemented with arginine. Cells were harvested in log phase (OD$_{750}$=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 4 mL of sterile TAP+40 µM sucrose. Cells (300 µL) were transferred to a sterile micro-centrifuge tube containing 300 mg of sterile glass beads. (0.425-0.6 mm, Sigma, USA) 100 µL of sterile 20% PEG 6000 (Sigma, USA) was added to the cells along with 1.5 µg of plasmid DNA. Prior to transformation, all the constructs were restriction digested either to linearize the construct or to excise the two collinear expression cassettes carrying selection marker and gene of interest together, from the plasmid backbone. Following addition of plasmid DNA, cells were vortexed for 20 seconds and plated on to TAP agar plates containing 50 μg/mL paromomycin and 100 μg/mL arginine.

For plasmid lacking any selection marker (pSSCR7 backbone), co-transformation was done. For co-transformation, CC424 strain was transformed using glass beads method following addition of the linearized target plasmid (3 µg DNA) and the plasmid harboring the Arg7 gene, p389 (1 µg DNA). Cells were plated on TAP agar plates without arginine.

4.2.8 Chlamydomonas Chloroplast transformation

Chlamydomonas chloroplast transformation was performed following the protocol described by Ishikura et al. (77). Briefly, the *psbA* deletion strain (CC741) of Chlamydomonas was grown in 100 mL of TAP liquid media. Cells were harvested in log
phase (OD
750=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 2 mL of sterile HS medium. Cells (300 µL) were spread in the center of HS agar plates. Gold particles (1µm) (InBio Gold, Eltham, Victoria, Australia) coated with plasmid DNAs were shot into Chlamydomonas cells on the agar plate using a Bio-Rad PDS 1000He Biolistic gun (Bio-Rad, Hercules, CA, USA) at 1100 psi under vacuum. Following shooting, cells were plated onto HS agar plates for selection.

4.2.9 PCR analysis

Genomic DNA was extracted from putative transformants growing on selection medium using a modified xanthine mini prep method described by Dr.Steve Surzycki (http://www.chlamy.org/methods/dna.html). A half loop of algal cells were resuspended in 300 µL of xanthogenate buffer (12.5 mM potassium ethyl xanthogenate, 100 mM Tris-HCl pH 7.5, 80 mM EDTA pH 8.5, 700 mM NaCl) and incubated at 65°C water for 1.0 hour. Following incubation, the cell suspension was centrifuged for 10 minutes (14,000 rpm) to collect the supernatant. The supernatant was transferred to a fresh micro-centrifuge tube and 2.5 volume of cold 95% ethanol (750 µL) was added. The solution was mixed well by inverting the tube several times allowing DNA to precipitate. The samples were then centrifuged for 5 min (14,000 rpm) to pellet the DNA. The DNA pellet was washed with 700 µL of cold 70% ethanol and centrifuged for 3.0 min. The ethanol was removed by decanting and the DNA pellet was dried using a speedvac to get rid of any residual ethanol. The DNA pellet was then resuspended in 100 µL of sterile double distilled water. 2-5 µL of the DNA sample was used as template for setting PCR.
4.2.11 Western blot analysis

Algal cells equivalent to one milliliters of OD\textsubscript{750}=1 from algal cultures were pelleted in micro-centrifuge tubes by centrifugation at 10,000 rpm for 1 minute at room temperature and resuspended in 50 µL of 1X Laemmli loading dye (0.063M Tris-HCl Ph 6.8, 2% SDS-PAGE, 10% glycerol, 5% B-mercaptaethanol, 0.05% bromo-phenol-blue), and incubated at 100°C for 5 minutes. Samples were removed from water bath, cooled to room temperature, centrifuged at 10,000 rpm for 2 minutes and the cell extract was separated on 10% SDS polyacrylamide gel by electrophoresis. Protein samples were transferred to PVDF membrane (Millipore, Billerica, MA, USA) by semi-dry blotting. The membrane was blocked with 0.5% BSA in TBS containing 0.05% Tween-20 for one hour at room temperature. For detection of P57 and P57-IHNV-G epitope fusions, anti P57 goat antibody conjugated to horseradish peroxidase (HRP) (KPL Inc, Gaithersburg, MD, USA) was used. Blots were incubated with the antibody at 1:1000 dilution for overnight at 4°C. For detection of IHNV-G protein, membrane was incubated with anti IHNV sheep antibodies (Microtek International Inc, Saanichton, BC, Canada) at 1:1000 dilution for overnight at 4°C. HRP conjugated Anti-sheep IgG antibody produced in donkey (Sigma, Saint Louis, MO, USA) was used as secondary antibody at 1:10,000 dilution. Detection of IHNV-G-luciferase fusion protein was done by incubating membrane with anti G-LUC rabbit polyclonal antibody (Nanolight technologies, Pinetop, AZ, USA) at 1: 1000 dilution at 4°C for overnight. Anti-rabbit IgG antibody (Sigma, Saint Louis, MO, USA) conjugated to horse-radish peroxidase was used as secondary antibody at 1:10000 dilution. In all cases the detection was completed by
chemiluminescence using luminol (0.4 mM) and iodophenol (8.0 mM) (Sigma, Saint Louis, MO, USA) (173).

4.2.12 Luciferase assays

Luciferase assays were performed at room temperature with POLARstar OPTIMA microplate luminometer (BMG Labtech, Germany) with automated substrate injection. For screening Chlamydomonas transformants for luciferase expression, transformants were inoculated in 2 mL 96 well (Nunc, Rochester, NY, USA) plates containing TAP media supplemented with arginine and grown for 2 to 3 days under continuous light. One hundred microliters of algal culture was diluted with 100 µL of TAP medium in 96 well plate and luminescence was recorded after automatic injection of 10 µL coelenterazine (0.1 mM in 10% ethanol) (NanoLight technologies, Pinetop, AZ USA) every 4 s for total of 24 s at the highest amplification and directly referred as luminescent units.

4.2.13 Vaccine trial on Rabbits

Selected algal transformants expressing the antigens were grown in 10 L of TAP medium up to log phase (OD750= 0.8 to 1.0), harvested by centrifugation and the cells were resuspended in 400 mL of fresh TAP media and shipped at 4°C to Antibodies Inc. (Davis, CA, USA). The Antibodies Inc. protocol for production of polyclonal antibodies in rabbits was followed. Rabbits were fed 4oz of algae twice a day on 1st, 14th, 21st, 35th and 49th day of the program. Test bleed was done on 28th day and production bleed was done on 57th day of the program. Presence of antibodies against the antigen of interest in
the immune serum from rabbits was detected using western blot or ELISA. Pre-immune serum was used a control for all the experiments.

4.2.14 ELISA Protocol

The Anaspec SenoLyte ADHP Horseradish peroxidase ELISA Assay kit (Anaspec, Fremont, CA, USA) was used for conducting ELISA experiments following manufacturer’s protocol. Briefly, wells of a 96 well ELISA plate (Nunc, Rochester, NY, USA) were coated with 1 µg of synthetic peptides by overnight incubation at 4°C. Uncoated well surfaces were blocked by incubation at room temperature for 60 minutes with 200 µL of blocking solution (PBS+1% BSA+ 0.02% Tween 20). Following blocking, wells were washed five times with wash buffer (PBS + 0.02% Tween 20). Pre-immune serum and Immune serum from rabbits was diluted to desired dilution in EIA buffer (PBS + 0.1% BSA + 0.02% Tween 20) and 100 µL was dispensed into the wells. Plate was incubated at room temperature for 90 minutes. Wells were then washed five times with wash buffer and incubated with 100 µL of HRP conjugated anti-rabbit antibody diluted (1:5000) in EIA buffer for 90 minutes at room temperature. Following incubation with secondary antibody, wells were washed five times and incubated with 100 µL of ADHP reaction mixture (0.5 µL ADHP + 1 µL H₂O₂ + 9.85 µL assay buffer, all supplied with kit) at room temperature for 30 minutes before measuring fluorescence in POLARstar OPTIMA microplate luminometer (BMG Labtech, Germany) with excitation at 530 nm and emission at 590 nm.
4.3 Results

4.3.1 Analysis of Phage sequences and IHNV-G protein sequence

Thirty plaques obtained following screening of dodecapeptide phage display library against anti-IHNV immune serum from trout were amplified and sequenced. Good sequence was obtained for 29 phages. Phage DNA sequences were translated into amino acid sequences and analyzed by aligning with G amino acid sequence using European bioinformatics institute (EBI) alignment tool (http://www.ebi.ac.uk/Tools/emboss/align/). The phage sequences showed good alignment with G amino acid sequence. The number of identical amino acids ranged from 2 to 7 while the number of similar amino acids ranged from 5 to 9 (Table 4.2) in alignments of phage sequences with G amino acid sequence. In many cases 2 to 4 phage sequences aligned to the same regions of G protein forming clusters (Figure 4.1). Most importantly 3 phage sequences aligned partially with the known linear epitope of G.

In order to determine the surface amino acid residues of the G protein, the G amino acid sequence was subjected to protein secondary structure prediction using Jpred (http://www.compbio.dundee.ac.uk/www-jpred/) (29) and Predict protein (http://www.predictprotein.org/) (137). The sequence was also subjected to hydropathy analysis using the Kyte-Doolittle algorithm (http://gcat.davidson.edu/DGBP/kd/kyte-doolittle.htm) (89) with a window size of 9 amino acids. G sequence was also subjected to Emboss antigenic peptide prediction algorithm analysis (http://liv.bmc.uu.se:16080/EMBOSS). Combined analysis of the results of protein secondary structure prediction, hydrophilicity plot and phage sequence analysis showed
that the phage sequences preferentially aligned with the predicted surface residues of the G protein (Figure 4.2). Based on this analysis and results of antigenic peptide prediction, 8 putative G protein epitopes were selected for testing (Table 4.3 & Figure 4.3). The length of the selected epitopes ranged from 17 to 19 amino acids.
Table 4.3: Analysis of alignment of phage sequences to G protein

Amino acid sequences of 29 phages picked following screening of phage library against immune serum of trout exposed to IHNV. The phage sequences were aligned to G protein sequence. The number of identical amino acids (marked in red) and similar amino acids (marked in blue) upon alignment of each phage sequence with G protein are also shown. The phage sequence forming alignment clusters are marked as a, b, c, d, e, f and g.

<table>
<thead>
<tr>
<th>Phage No.</th>
<th>Sequence</th>
<th>No. of Identical aa</th>
<th>No. of Similar aa</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>DNPIPQGLALP</td>
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<td>7</td>
</tr>
<tr>
<td>P-2</td>
<td>SSVTPHPWPRPV</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>P-3</td>
<td>KAYVSRPPGMH</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>P-4</td>
<td>APHTWRITGMH</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>P-5</td>
<td>QGMLLEAPLAP</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>P-6</td>
<td>TGHMQPRDAGHA</td>
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<td>6</td>
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<tr>
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<td>VPVMVDRGGSA</td>
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<tr>
<td>P-8</td>
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<td>8</td>
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<tr>
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<td>SSDSNTSHGMGP</td>
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<td>5</td>
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</table>
Figure 4.1: Alignment of phage sequences to IHNV-G protein sequence.

The regions of G protein showing alignment with phage sequences are marked with arrows. A number of phage sequences aligned to the same regions of G protein forming clusters.
Figure 4.2: Kyte-Doolittle hydrophobicity plot of G protein.

The regions with values of more than 0.0 represent the hydrophobic regions of the protein and regions with less than 0.0 are the hydrophilic regions of the protein. The window size used for the plot is 9 amino acids. The phage sequences aligned (shown in pink) mostly to the hydrophilic regions of the protein.
Figure 4.3: Putative IHNV-G epitopes selected as vaccine candidates.

The regions of G protein showing alignment with phage sequences are marked with arrows. The antigenic peptides picked by Emboss antigenic peptide prediction algorithm are represented in blue. The putative G peptides selected as vaccine candidates based on all the analysis are shown in boxes.
Table 4.4: Amino acid sequences of the putative IHNV-G epitopes selected as vaccine candidates.

<table>
<thead>
<tr>
<th>Epitope No.</th>
<th>Epitope sequence</th>
<th>Location in IHNV-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LITCGANSQTVQPDTASES</td>
<td>13 - 31</td>
</tr>
<tr>
<td>2</td>
<td>SNRVVKATSYEHHHPWGLG</td>
<td>227 - 244</td>
</tr>
<tr>
<td>3</td>
<td>STNSVTPYLLSKFRSPH</td>
<td>320 - 336</td>
</tr>
<tr>
<td>4</td>
<td>SIKSVPHPSILAFYNETDV</td>
<td>422 - 440</td>
</tr>
<tr>
<td>5</td>
<td>TLGGIPLVLLLAIAACY</td>
<td>466 - 482</td>
</tr>
<tr>
<td>6</td>
<td>TYRAHHATNFTKWERPFG</td>
<td>371 - 388</td>
</tr>
<tr>
<td>7</td>
<td>SESREECLEAHAEIISTNS</td>
<td>307 - 325</td>
</tr>
<tr>
<td>8</td>
<td>TCDFSQEIKGHLFGDKIS</td>
<td>212 - 228</td>
</tr>
</tbody>
</table>
4.3.2 Expression of p57-G epitope fusion protein in Chlamydomonas

The eight putative G protein epitopes were fused to the C-terminus of p57 protein from *Renibacterium salmoninarum* by PCR using overlapping primers and cloned into vector pBA155 (108) creating plasmids pCVAC6, pCVAC7, pCVAC8, pCVAC9, pCVAC10, pCVAC11, pCVAC12 and pCVAC13 respectively. The p57 protein is a strong immunogen and was used as a carrier protein to aid in stimulating immune response in fish. Chloroplast transformation of CC741 strain of Chlamydomonas was performed with the p57-G epitope constructs using particle bombardment. Colonies were selected based on their ability to carry out photosynthesis and survive on HS medium. Integration of the transgenes into chloroplast genome was confirmed by PCR. The PCR reaction did not work for any of the pCVAC6 clones while 50 to 70% of clones from other constructs were PCR positive. Expression of p57-G epitope fusion protein was confirmed in PCR positive clones by immunoblot analysis using anti-p57 antibody. In all the clones with different constructs a ~60kDa band representing p57-G epitope fusion protein was seen (Figure 4.4, 4.5 and 4.6). In addition to the 60kDa band, two more bands of ~35kDa and ~30kDa were seen in all the transformants. These bands most probably represent the degradation products of the p57-G epitopes fusion protein.
Figure 4.4: Immunoblot analysis of PCR positive CC741/pCVAC7 and CC741/pCVAC8 clones.

A) Immunoblot analysis of PCR positive CC741/pCVAC7 and B) CC741/pCVAC8 clones to confirm expression of p57-IHNV G epitope fusion protein. The presence of 60 kDa band among transformants confirms the expression of p57-IHNV G epitope fusion protein. Dual color precision plus protein ladder was used. Blot was probed with anti p57 antibody.
Figure 4.5: Immunoblot analysis of PCR positive CC741/pCVAC9, CC741/pCVAC10, CC741/pCVAC11 and CC741/pCVAC12 clones.

A) CC741/pCVAC9 and CC741/pCVAC10 clones and B) CC741/pCVAC11 and CC741/pCVAC12 clones. The presence of the 60 kDa band among transformants confirms the expression of p57-IHNV G epitope fusion protein. Dual color precision plus protein ladder was used. Blot was probed with anti p57 antibody.
Figure 4.6: Immunoblot analysis of PCR positive CC741/pCVAC13 clones.

The presence of the 60 kDa band among transformants confirms the expression of p57-IHNV G epitope fusion protein. Dual color precision plus protein ladder was used. Blot was probed with anti p57 antibody.
4.3.3 No immune reaction was observed in rabbits vaccinated with transgenic Chlamydomonas expressing p57-IHNV-G epitopes.

Chlamydomonas chloroplast transformants expressing p57-IHNV-G fusion protein were divided into two groups. Group one consisted of pCVAC7, pCVAC8 and pCVAC9 and group two consisted of pCVAC10, pCVAC11, pCVAC12 and pCVAC13. These two groups of algae were fed to rabbits to test whether the selected epitopes can induce any kind of immune response. The groups of algae were grown and shipped to Antibodies Inc. (Davis, CA, USA) for feeding the rabbits. The Antibodies Inc. regime for production of polyclonal antibodies in rabbits was followed. 4oz of algae were fed to rabbits twice a day on 1st, 14th, 21st, 35th, and 49th day of the program. Rabbits were bled for test and production on 28th and 57th day respectively. Immune serum from the rabbits was used to carryout immunoblot and ELISA analysis to test for presence of any anti IHNV-G epitope antibodies in the immune serum. For all the experiments pre-immune serum was included as control. ELISA experiment was carried out on synthetic peptides representing the seven different G protein epitopes. Wells were coated with 1.0µg of synthetic peptides and probed with three different dilutions of the immune serum (1:100, 1:300 and 1:1000). No specific immune response was observed against any of the peptides (Figure 4.7). In immunoblot experiments cell lysate of E. coli cells expressing G-Tobacco mosaic virus coat protein (TMVCP) fusion protein and G-Maltose binding protein (MBP) fusion protein were probed with the immune serum from rabbits. The immune serum from rabbits failed to detect the G-TMVCP and G-MBP fusion proteins.
(Figure 4.8) indicating that either the serum does not contain any antibodies against the IHNV-G epitopes or they fail to recognize the IHNV-G protein.
Figure 4.7: ELISA analysis of immune-serum from rabbits vaccinated orally by feeding transgenic algae expressing p57-IHNV-G epitope fusions.

Wells were coated with 1µg of peptides representing IHNV-G epitopes. Immune serum was used at 1:1000 dilution.
Figure 4.8: Immunoblot analysis of Immune serum from rabbits vaccinated with transgenic algae expressing p57-IHNV-G epitopes.

Western blot analysis to test for presence of anti IHNV-G epitope antibodies in the immune serum from the two rabbits fed with transgenic algae expressing p57-IHNV-G epitope fusions. Blots probed with pre-immune serum and immune serum from rabbit fed with first (A) and second (B) group of transgenic algae. Loading pattern: 10µL of cell lysate from uninduced G-TMVCP fusion (Lane 1), 10µL of cell lysate from induced G-TMVCP fusion (Lane 2), 10µL of cell lysate from uninduced G-MBP protein fusion (Lane 3) and 10µL of cell lysate from induced G-MBP protein fusion (Lane 4). Dual color precision plus protein ladder was used.
4.3.4 Expression of IHNV-G in Chloroplast transformants could not be confirmed

Codon optimized IHNV-G sequence for Chlamydomonas chloroplast expression was assembled using overlapping primers following the oligo shuffling protocol described by Stemmer et al. (154), and cloned into vector pBA155 creating plasmid pCVAC21. CC741 strain of Chlamydomonas was transformed and colonies growing on selection medium were screened by PCR to confirm integration of the transgene into the chloroplast genome. Immunoblot experiments were carried out to check the expression of G protein among PCR positive chloroplast transformants using anti-His tag antibodies and anti-IHNV antibodies. While no band in the size range of G protein (~55kDa) was seen with anti-His tag antibodies (Figure 4.9), a thick non-specific band in the size range of 50-55 kDa was seen when the blot was probed with anti-IHNV antibody (Figure 4.9) making it impossible to conclude anything regarding expression of G protein among these clones. Attempts were made to resolve the G protein band from non-specific bands by running long gels; however this strategy was not effective in getting rid of the non-specific bands. Since we were not able to confirm the expression of G protein, RT-PCR reactions were set to test for the presence of G transcripts. The RT-PCR reactions worked (Figure 4.10) indicating that the G coding sequence was getting transcribed. Since the clones were RT-PCR positive, a custom G protein peptide antibody was made (GenScript, Piscataway, NJ, USA). The peptide anti G protein antibody was used in immunoblot experiments to test the expression of G protein among RT-PCR positive clones. However, even after repeating experiments for several times, we failed to detect expression of G-protein (Figure 4.11). Therefore, we were unable to confirm whether
these RT-PCR positive clones were expressing IHNV-G protein. Hence, these clones were not used for any further experiments.
Figure 4.9: Western blot analysis of PCR positive CC741/pCVAC21 (codon optimized IHNV G for Chlamydomonas chloroplast expression) clones.

A) Blot probed with anti-His antibodies. The IHNV G protein band (55 kDa) was not detected. B) Blot probed with anti-IHNV antibodies. A thick non-specific band in the size range of 55-60 kDa was seen which probably masks the IHNV G protein band. Dual color precision plus protein ladder was used.
Figure 4.10: RT-PCR analysis of CC741/pCVAC21 clones.

Gene specific primers were used for cDNA synthesis. Products of RT-PCR reaction were loaded in lanes 1 through 4 while products of control PCR reaction, set to test contamination of RNA samples with DNA were loaded in lanes 5 through 8. CC741 (lane 1 & 5) and CC741/pCVAC17 clone 4 (lane 5 & 8) expressing p57 protein were included as negative and positive controls respectively. CC741/pCVAC21-1 (lanes 2 & 6) and CC741/pCVAC21-2 (lanes 3 & 7) were tested for presence of IHNV-G transcripts. 1kb plus DNA ladder was used as marker.
Figure 4.11: Western blot analysis of PCR positive CC741/pCVAC21 clones.

Blot probed with Anti G peptide antibody. No band corresponding to the size of G protein (50kDa) was seen in any of the clones. The 70 kDa G-TMV CP fusion band was seen in positive control indicating that the antibody was working. Dual color precision plus protein ladder was used. The two pink bands in the ladder represent bands of 25 kDa and 75 kDa in size.
4.3.5 Expression of G protein by nuclear transformants

IHNV-G coding sequence, optimized for Chlamydomonas nuclear expression was assembled using the oligo shuffling method of Stemmer et al. (154). CC424 strain of Chlamydomonas was transformed and clones were screened by PCR for the presence of G coding sequence. PCR positive clones were subjected to immunoblot analysis to check expression of G protein using anti-His antibody. The 55 kDa G protein band was not seen in any of the clones. These PCR positive clones were then subjected to RT-PCR analysis to check for presence of G transcripts. Transcript of G was not detected in any of the PCR positive clones. This meant that these clones were not transcribing G coding sequence and hence they were discarded.

Since we observed good expression of the reporter protein Gaussia luciferase by Chlamydomonas, it was decided to make N-terminal and C-terminal fusions of luciferase with G protein to allow for easy screening of clones and to promote stability and proper folding of G protein. Moreover, since luciferase is secreted by the cell wall mutant strain CC424 into the medium, we expected that fusion of luciferase to G protein will guide the G protein through the secretary pathway allowing post-translational modifications such as glycosylation to occur. Such modifications are very important and in many cases contribute towards antigenicity of the protein. Hence luciferase was fused to the N-terminus and C-terminus of G creating plasmids pCVAC161 and pCVAC122 respectively. CC424 strain was transformed with these constructs and paromomycin resistant colonies were screened for luciferase expression. 400 colonies from each construct were screened for luciferase activity. Colonies showing luciferase activity were
subjected to immunoblot analysis using anti-luciferase antibodies. While two clones from pCVAC161 construct (Figure 4.12) showed presence of the 70kDa LUC-G band, only one clone from pCVAC122 construct showed presence of 70kDa band only from the medium fraction of the culture (Figure 4.12). Immunoblot analysis of these clones was repeated with anti-G peptide antibody. The 70 kDa band was seen in all the three clones (Figure 4.13). This confirmed the expression of G-LUC fusion protein among these clones. The three selected clones were used for oral vaccine trial on rabbits to check whether they can act as oral vaccine and stimulate the immune response in rabbits.
Figure 4.12: Immunoblot experiments to confirm expression of IHNV-G-LUC fusion protein by CC424/pCVAC122 and CC424/pCVAC161 clones.

A) Total soluble protein extract from cells equivalent to 1.0 mL of culture at OD$_{750}$=1 was loaded for each sample for the cell fraction samples. Total soluble protein extract from supernatant of 1.0ml of OD$_{750}$=1 culture was loaded for each sample for the supernatant samples. B) Total soluble protein extract from cells equivalent to 1.0 ml of OD$_{750}$ = 1 was loaded for each sample Blots were probed with anti-LUC antibody. Dual color precision plus protein ladder was used. The two pink bands in the ladder represent bands of 25 kDa and 75 kDa in size.
Figure 4.13: Immunoblot experiment on selected CC424/pCVAC122 (G-luciferase) and CC424/pCVAC161 (Luciferase-G) clones.

Immunoblot experiment with selected CC424/pCVAC122 and CC424/pCVAC161 clones showing expression of G-LUC fusion protein. Total soluble protein extract from cells equivalent to 1.0 mL of OD$_{750}$=1 was loaded for each sample. The 70 kDa G-luciferase fusion protein is seen in all the clones. Blot was probed with anti G peptide antibody. Dual color precision plus protein ladder was used.
4.3.6 Feeding of Chlamydomonas transformants expressing IHNV-G-LUC fusion protein stimulates immune response in rabbits.

Transformants expressing G-LUC fusion protein were fed to rabbits to test if they can function as oral vaccines and stimulate immune response in rabbits against the G protein. Algae were grown and shipped to Antibodies Inc. (Davis, CA, USA) for feeding to rabbits. The Antibodies Inc. regime for production of polyclonal antibodies in rabbits was followed. 4 oz of algae were fed to rabbits twice a day on 1st, 14th, 21st, 35th, and 49th day of the program. Test bleed and production bleed were done on 28th and 57th day respectively. Since immunoblots containing total protein extract from Chlamydomonas cells with immune serum from rabbits produced large number of non specific bands, cell lysate of BL21(DE3) cells expressing IHNV G-tobacco mosaic virus coat protein (TMVCP) fusion was probed with the immune serum from the rabbits in immunoblot experiments to test for presence of anti G protein antibodies in the serum. Immune serum from rabbits pCVAC122-203 and pCVAC161-83 produced clear bands in the expected size range (60 to 70 kDa) indicating that the antibodies present in the immune serum were able to detect the IHNV-G protein in immunoblot experiments (Figure 4.14). Even though the size of the expected band is 70kDa, we have observed multiple bands of different sizes when the G-TMVCP fusion was probed with anti IHNV G antibodies (Figure 4.11). This implies that the 60kDa band observed with the immune serum from rabbit pCVAC122-203 is most likely G-TMVCP fusion band. With the immune serum of rabbit CC424/pCVAC161-58 also a band of ~70kDa was observed. However, the result
was not very convincing as a faint band of same size was also observed with the pre-immune serum.

Results of the immunoblot experiments indicate that the Chlamydomonas transformants expressing G-LUC fusion protein were successful in inducing specific immune response in rabbits against the IHNV-G protein and can be used as oral vaccines. These clones will now be used to test whether they can induce specific immune response against the G protein in trout fries through oral or immersion vaccination.
Figure 4.14: Immunoblot experiment to detect IHNV G protein with immune serum from rabbits orally vaccinated with Chlamydomonas cells expressing IHNV G-luciferase fusion protein.

One microgram cell lysate of BL21(DE3) expressing G-TMVCP fusion was probed with pre-immune serum (PI) and immune serum (I) from rabbits vaccinated with transgenic Chlamydomonas clones expressing G-luciferase fusions. The CC424/pCVAC122 clones express G-luciferase fusion protein, while the CC424/pCVAC161 clones express Luciferase-G fusion proteins. Dual color precision plus protein ladder was used.
4.4 Discussion

In this work, we have tried to develop the unicellular alga, *Chlamydomonas reinhardtii* as a system to deliver vaccines to fish against IHNV. Glycoprotein (G), found on the surface of IHNV particles is known to induce production of neutralizing antibodies in rabbits and fish (50). Oral and immersion vaccination of fish with purified G protein provides effective protection (35% to 83% more survival over control) (50). However, production and purification of G protein significantly increases vaccination cost. Therefore, an attempt to develop an effective vaccine delivery system that minimizes costs associated with vaccine production and delivery was made. Two strategies to develop algal based vaccines against IHNV were evaluated. The first strategy was an epitope based strategy that involved identification of putative G protein epitopes and expression of selected epitopes as C-terminus fusions with the p57 protein of *R. salmoninarum* in Chlamydomonas chloroplast. Attempts have been made in past to map the neutralizing epitopes of G protein using monoclonal antibodies (73, 74, 171). Huang et al. (73) mapped a linear epitope spanning from amino acid 230 to 238 and a conformational epitope consisting of amino acids 78 to 81 and 272 to 276 of G protein. Xu et al. (171) also showed that fish vaccinated with lysate of bacterial cells expressing fragments of G protein provide effective protection to fish (171), indicating that perhaps the G protein harbors several neutralizing linear epitopes and glycosylation of G protein is not essential for inducing protective immunity. Moreover, of the 30 monoclonal antibodies used for characterizing G neutralizing epitopes by Huang et al. (74), all 30 were able to detect native and deglycosylated G protein and 9 were able to detect reduced
G protein in immunoblot experiments, signifying that there are probably several neutralizing conformational and linear epitopes of IHNV-G and the antibodies specifically recognize carbohydrate free epitopes. These findings suggest that there is great potential for developing an epitope based vaccine for IHNV. Therefore, we screened a dodecapeptide phage display library against trout anti-IHNV immune serum to identify phage sequences that mimic IHNV-G epitopes. Importantly, our screen was able to pick the known linear epitope of G protein as 3 phage sequences showed alignment with it. As expected, the G protein conformational epitope was not picked. All the remaining phages aligned to the predicted surface residues of the G protein. Transgenic Chlamydomonas expressing putative G protein epitopes fused to C-terminus of p57 were used to vaccinate rabbits orally and the immune serum from rabbits was analyzed for presence of anti-G protein antibodies by ELISA and immunoblot. However, we failed to detect synthetic peptides representing the putative epitopes in ELISA or the G protein in immunoblots with the immune serum from rabbits. This indicates that either the selected epitopes did not induce any immune response in rabbits or the antibodies in the immune serum could not recognize the synthetic peptides or G protein. The result was not surprising as there are no success stories as far as epitope based vaccines are concerned. Although several epitope based vaccines against HIV, Hepatitis C and Influenza virus are in advanced stages of trials (2, 78, 132, 176). Previous attempts of intraperitoneal injection of trout with synthetic peptides representing putative G protein epitopes conjugated with bovine serum albumin and mixed with adjuvant had also failed to provide any protective immunity (49). Incorrect epitope prediction, use of inappropriate
carrier molecule and lack of T-cell stimulation by the selected peptides are probably the possible reasons for failure of putative G protein epitopes to induce immune response in the present and earlier studies.

The second strategy involved expression of G protein in Chlamydomonas. Since G protein is known to induce production of neutralizing antibodies in fish, there was very high possibility that oral vaccination of rabbits with transgenic Chlamydomonas expressing G protein would lead to induction of G protein specific immune response in rabbits. Moreover, earlier reports of successful vaccination of fish with cell lysate from bacterial cells expressing fragments of G protein (171) supported this strategy. Immune serum from rabbits fed with transgenic Chlamydomonas expressing LUC-G protein fusions was tested for presence of anti-G antibodies by immunoblot and the results were encouraging. The rabbit immune serum was able to detect G-TMVCP protein fusions in the immunoblots indicating that this strategy can work. In the next stage, the transgenic Chlamydomonas expressing LUC-G protein fusions will be used to conduct a vaccination trial on fish. Again the prospects of this strategy working with fish are high because similar studies involving oral vaccination of salmonid fish with yeast cells expressing viral capsid protein of infectious pancreatic necrosis (IPN) virus were able to induce specific immune response in fish (4).

In summary, we have shown that feeding of transgenic Chlamydomonas expressing IHNV G protein to rabbits induces specific immune response in rabbits against G protein. These transgenic algae will now be used to carryout a vaccination trial on fish. However the other strategy employing putative G protein epitopes for
vaccination did not work, indicating that more effective strategies and algorithms need to be developed to facilitate identification of epitopes correctly and efforts also need to be made for identification of T-cell epitopes.
CHAPTER 5: DEVELOPMENT OF CHLAMYDOMONAS REINHARDTII AS A SYSTEM TO CONTROL MOSQUITOES

5.1 Introduction

The importance of controlling mosquito populations for the benefit of human health is universally known and accepted worldwide. Mosquitoes transmit many life threatening parasitic and viral diseases including malaria (Anopheles sps), filariasis (Culex, Mansonia and Anopheles sps), yellow fever (Aedes aegypti), and dengue fever (Aedes aegypti). In 2008, about 247 million cases of malaria alone were reported from Africa resulting in nearly one million deaths and accounting for nearly 20% of childhood mortality in Africa (WHO fact sheets http://www.who.int/mediacentre/factsheets/fs094/en/index.html). Various strategies have been tried to control mosquito populations in the last 100 years, including use of chemical pesticides and biological control agents (entomophagous bacteria, fungi, viruses, parasites and predators). In addition to effecting human health and harmful effects on the environment, the indiscriminate use of chemical pesticides has led to development of resistance among mosquito populations (126). While the biological control agents are safe, environment friendly and host specific they have not been very
effective due to difficulties associated with maintenance and multiplication of their populations in natural mosquito habitats (126). Hence various new strategies involving application of recombinant DNA technologies are under evaluation. These include development of transgenic mosquitoes that are sterile or unable to transmit parasites or carry selfish genes such as Medea elements and homing endonuclease genes (27). These strategies are based on the premise that release of transgenic mosquito populations into the natural breeding grounds of mosquitoes would result in replacement of the wild-type population by transgenics after few generations of mating between the transgenic and wild-type mosquitoes. However, no data is available regarding the impact of releasing transgenic mosquitoes into the environment or effectiveness of these strategies.

*Bacillus thuringiensis* ssp. *israelensis* (Bti) and *Bacillus sphaericus* (Bs) are the two species of Bacillus that are known to produce parasporal crystalline inclusion bodies that are toxic to mosquito larvae (13, 64) and are very popular biological control agents. Spore preparations of Bti and Bs are available as commercial formulations for mosquito control (126). However, application of these spores have some limitations, such as sedimentation of spores out of larval feeding zone (81) and inactivation of toxins by UV (91). One of the ways to overcome these problems is to express the toxin proteins of Bti and Bs in aquatic bacteria and algal species that live in the feeding zone of mosquito larvae and serve as food source for the larvae. Hence, attempts have been made to express Cry genes from Bti and Bs among cyanobacterial species of Anabaena and Synechococcus (7, 97, 139, 151, 170) and several aquatic bacterial species (175). The transgenic cyanobacteria and aquatic bacteria expressing Cry toxins have been found to
be toxic to mosquito larvae (7, 97, 139, 151, 170). We propose that the micro-alga *Chlamydomonas reinhardtii* would serve as a valuable addition to the list of aquatic microorganisms that are being engineered for mosquito control. Chlamydomonas was selected because 1) it can be genetically manipulated 2) being an alga it grows on stagnant water, which serve as breeding grounds for mosquitoes, 3) unicellular algae is the natural food for mosquito larvae 4) it can grow photosynthetically and 5) has low cost of production.

We have tried two strategies to develop Chlamydomonas into mosquito larvicide. The first strategy involved expression of the engineered *Cry4Ba* protein from Bti by Chlamydomonas nuclear or chloroplast genome. Engineered *Cry4Ba* was selected because while the native *Cry4Ba* endotoxin is toxic only to *Anopheles* and *Aedes*, mosquito genera (38), the engineered *Cry4Ba* is also toxic to *Culex* mosquito (1).

The second strategy involved controlling mosquito populations by silencing genes essential for mosquito survival by feeding transgenic Chlamydomonas expressing duplex RNAs (dsRNA) targeting these genes to mosquito larvae. This strategy has several advantages like 1) it does not involve expression of foreign proteins, which can be an issue 2) the RNAi elements are very specific, inhibiting expression of gene(s) of interest and 3) only a small amount of dsRNA of target gene is required as a trigger to initiate production of RNAi elements like siRNAs by the evolutionarily conserved gene silencing machinery. Several studies have shown that both mosquitoes and Chlamydomonas possess the RNAi machinery (13, 16, 118, 141). The evidence in support of this strategy
for biological control comes from studies showing development of nematode resistance in plants producing dsRNAs targeting essential nematode genes (75, 172).

The mosquito gene 3-hydroxykynurenine transaminase (3HKT) was selected as mosquito-specific target for inactivation. 3HKT catalyzes the transamination of reactive 3-hydroxykynurenine (3HK) to chemically stable xanthurenic acid (XA) in the tryptophan metabolic pathway of mosquitoes (67). 3HK is a highly reactive intermediate in the tryptophan metabolic pathway that can easily undergo oxidation under normal physiological conditions resulting in production of reactive oxygen species that can have lethal consequences to organisms (116, 117). In fact injection of µM amounts of 3HK have been found to induce apoptosis of neurons leading to paralysis in flies (165). Hence 3HK levels are very tightly regulated by its immediate conversion into more stable downstream intermediates of tryptophan pathway (67). While 3HK is converted by Kynurenine aminotransferases (KAT) in humans and mammals to stable intermediates, mosquitoes have evolved 3HKT to prevent accumulation of 3HK (67). We selected 3HKT as target because till date, this protein has only been found among various genera of mosquitoes and has not been found among other organisms including other dipterans such as Drosophila, whose genomes has been sequenced. Moreover 3HKT is an attractive target because it is expressed during all the active feeding stages (larvae and adults) of mosquito life cycle (136). *Anopheles gambiae* 3HKT was selected as target. A 328 bp region of *Anopheles* 3HKT that shows 70% identity with *Aedes* and *Culex* 3HKT and does not show alignment of more than 11 contiguous bases to sequences from other organism in NCBI database was selected for making inverted repeat constructs.
While there were issues with expression of Cry4Ba in Chlamydomonas, the RNAi strategy involving 3HKT was found to be effective in reducing the fitness of mosquito larvae.

5.2 Materials and Methods

5.2.1 Algal strains and cultural conditions

Chlamydomonas strains CC424 (cw15, arg2, sr-u-2-60 mt-) and CC741 (ac-u- (beta) mt+) were obtained from the Chlamydomonas culture collection at Duke University, USA. Strains were grown mixotrophically in liquid or on solid TAP Medium (69) at 23\(^0\) C under continuous white light (40 \(\mu E m^{-2} s^{-1}\)), unless otherwise stated. Medium was supplemented with 100 \(\mu g/mL\) of arginine when required. Selection of nuclear transformants was performed by using solid TAP medium or TAP medium supplemented with 100 \(\mu g/mL\) of arginine and 50 \(\mu g/mL\) of paromomycin or 25 \(\mu g/mL\) of hygromycin. Selection of chloroplast transformants using strain CC741 (ac-u-(beta) mt+) was performed with high salt (HS) medium.

5.2.2 Vector Construction

Engineered Cry4Ba (R203A, D545PAT) (1) was kindly provided by Dr. Donald Dean (Ohio State University, Columbus, USA). Cry4Ba was amplified using primers CryFwd1 (5’- TATTACCATGGGCAATTCAAGGTATCCGGGTGATGAC-3’) and CryRev1 (5’- ATAATGCAATGCTACTCTCATGCAAAATTTAGCTTTCAATG CTTTCG -3’), introducing NcoII and SphI sites at 5’ and 3’ ends, respectively. Cry4Ba coding sequence was digested with NcoI/SphI and cloned into NcoI/SphI sites of pGatpA vector (77) creating plasmid pCry1. The 3.4kb Cry4Ba coding sequence was excised as
XhoI/SphI fragment along with a portion (about 100 bp) of atpA promoter and cloned into same sites of vector pBA155 creating plasmid pCry4. For cloning into Chlamydomonas nuclear transformation vector pSSCR7 (147) Cry4Ba was amplified with primers CryFwd2 (5’-TATTACATATGAATTAGCTATCCGTTAGCGAATGAC-3’) and CryRev2 (5’-ATAATAAAGCTTCCACTCGTCATGCAAATTAATTCAATG CTTCG-3’) and cloned into NdeI/HindIII sites of pSSCR7.

For making 3HKT inverted repeat (IR) constructs, 328 bp long fragment of Anopheles gambiae 3HKT coding sequence (Gen Bank accession number AM042695.1) representing the region from 952 to 1253 bp of 3HKT gene was amplified with primers HKTFwd2 (5’-ATGCTAAGCTTGCATGCATGAACCAAAACGTTATCATGAC-3’) and HKTRev2 (5’-AAGATGGATCCGCTAGCATAATACCCACACGCATGAC-3’) and cloned into HindIII/BamHI sites of vector pBSKS creating plasmid pCVAC88. To create 3HKT IR, 3HKT amplified with primers HKTFwd3 (5’-AGTCAGAGCTCCCATGGATGAACCAAAACGTTATCATGAC-3’) and HKTRev2 was cloned into SacI/XbaI sites of vector pCVAC88 creating plasmid pCVAC99. HKT inverted repeat from pCVAC99 was excised as NcoI/SphI fragment and cloned into same sites of vector pGatpA creating plasmid pCVAC101. Finally, the 3HKT inverted repeat from pCVAC101 was excised as XhoI/SphI fragment and cloned into XhoI/SphI sites of vector pBA155 creating plasmid pCVAC108.

For constructing Chlamydomonas nuclear transformation vectors carrying 3HKT IRs, 328bp long HKT fragment was amplified with primers HKTFwd4 (5’-
ATTAGCGGCGCCCATATGAACCAAAAACGTTATCACCATACT-3’) and HKTRev3 (5’- AATAAACTAGTCTGCAGATAATACCCACACGCATGC-3’) and cloned into NdeI/PstI sites of vector pCVAC135 creating plasmid pCVAC146. 3HKT amplified with primers HKTFwd4 and HKTRev3 was digested with NotI/SpeI restriction enzymes and cloned into same sites of pCVAC146 creating 3HKT IR. The new vector was designated as pCVAC150. To create 3HKT inverted repeat with intron as spacer between the repeat regions, 3HKT amplified with primers HKTFwd4 and HKTRev3 was cloned into NdeI/PstI sites of vector pCVAC131 creating plasmid pCVAC147. Actin intron1 amplified with primers ActIntron1Fwd2 (5’-ATTATATGCATGTGAAGGTGAGCAGGTGTTCAGGGCGC-3’) and ActIntron1Rev1 (5’-TAAGATACTAGTACGTGCCTGACGACCCGACGAC-3’) was digested with NsiI/SpeI and cloned into PstI/SpeI sites of pCVAC147 creating plasmid pCVAC148. The 3HKT fragment amplified with primers HKTFwd4 and HKTRev3 was then cloned into NotI/SpeI sites of vector pCVAC148 to create 3HKT inverted repeat. The new plasmid was named pCVAC153. To drive bidirectional transcription of 3HKT, 3HKT amplified with primers HKTFwd4 was cloned downstream of psaD promoter in NdeI/pstI sites of vector pSL18 creating plasmid pCVAC143. Chlamydomonas Actin promoter was amplified with primers ActinFwd4 (5’-ATCTATCTAGGATGCATGCGCTCCACGCATTAG-3’) and ActinRev3 (5’-AAGATCTGCAGCATATGTTTTGAATCTGTGCAGGCTCACGTCCGC-3’) was then cloned into PstI/XbaI sites of vector pCVAC143 creating plasmid pCVAC145.
5.2.3 Nuclear transformation of *C. reinhardtii*

*Chlamydomonas reinhardtii* nuclear transformation was performed using the glass bead method (85). Briefly, CC424 strain of Chlamydomonas was grown in 100 mL of TAP liquid media supplemented with arginine. Cells were harvested in log phase (OD$_{750}$=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 4 mL of sterile TAP+40 µM sucrose. Resuspended cells (300 µL) were transferred to a sterile micro-centrifuge tube containing 300 mg of sterile glass beads. (0.425-0.6 mm, Sigma, USA) Sterile 20% PEG 6000 (100 µL) (Sigma, USA) was added to the cells along with 1.5 µg of plasmid DNA. Prior to transformation, all the constructs were restriction digested either to linearize the construct or to excise the two expression cassettes carrying selection marker and gene of interest together, from the plasmid backbone. Following addition of plasmid DNA, cells were vortexed for 20 seconds and plated on to TAP agar plates containing 50 µg/mL paromomycin and 100 µg/mL arginine or 10 µg/mL hygromycin and 100 µg/mL arginine.

For plasmid lacking any selection marker (pSSCR7 backbone), co-transformation was done. For co-transformation, CC424 strain was transformed using glass beads method following addition of the linearized target plasmid (3 µg DNA) and the plasmid harboring the *ARG7* gene, p389 (1 µg DNA). Cells were plated on TAP agar plates without arginine.

5.2.4 Chlamydomonas chloroplast transformation

Chlamydomonas chloroplast transformation was performed following the protocol described by Ishikura et al. (77). Briefly, *psbA* deletion strain (CC741) of
Chlamydomonas was grown in 100 mL of TAP liquid media. Cells were harvested in log phase (OD$_{750}$=0.8 to 1.0) by centrifugation at 4000 rpm and resuspended in 2 mL of sterile HS medium. About 300 µL of cells were spread in the center of HS agar plates. Gold particles (1 µm) (InBio Gold, Eltham, Victoria, Australia) coated with plasmid DNAs were shot into Chlamydomonas cells on the agar plate using a Bio-Rad PDS 1000He Biolistic gun (Bio-Rad, Hercules, CA, USA) at 1100 psi under vacuum. Following shooting, cells were plated onto HS agar plates for selection.

5.2.5 PCR analysis

Genomic DNA was extracted from putative transformants growing on selection medium using a modified xanthine mini prep method described by Dr.Steve Surzycki (http://www.chlamy.org/methods/dna.html). A half loop of algal cells were resuspended in 300 µL of xanthogenate buffer (12.5 mM potassium ethyl xanthogenate, 100 mM Tris-HCl pH 7.5, 80 mM EDTA pH 8.5, 700 mM NaCl) and incubated at 65°C water for 1.0 hour. Following incubation, the cell suspension was centrifuged for 10 minutes (14,000 rpm) to collect the supernatant. The supernatant was transferred to a fresh micro-centrifuge tube and 2.5 volume of cold 95% ethanol (750 µL) was added. The solution was mixed well by inverting the tube several times allowing DNA to precipitate. The samples were then centrifuged for 5 min (14,000 rpm) to pellet the DNA. The DNA pellet was washed with 700 µL of cold 70% ethanol and centrifuged for 3.0 min. The ethanol was removed by decanting and the DNA pellet was dried using a speedvac to get rid of any residual ethanol. The DNA pellet was then resuspended in 100 µL of sterile
double distilled water and 2-5 µL of the DNA sample was used as template for setting PCR.

5.2.6 Immunoblot blot analysis

Cells equivalent to one milliliters of OD$_{750}$=1 from algal cultures were pelleted in microcentrifuge tubes by centrifugation at 10,000 rpm for 1 minute at room temperature and resuspended in 50 µl of 1X Laemmli loading dye (0.063M Tris-HCl pH 6.8, 2% SDS-PAGE, 10% glycerol, 5% B-mercaptaethanol, 0.05% bromo-phenol-blue), and incubated at 100°C for 5 minutes. Samples were removed from water bath, cooled to room temperature, centrifuged at 10,000 rpm for 2 minutes and the cell extract was separated on 10% SDS polyacrylamide gel by electrophoresis. Protein samples were transferred to PVDF membrane (Millipore, Billerica, MA, USA) by semi-dry blotting. Membrane was incubated with anti Cry4Ba rabbit polyclonal antibody, kindly provided by Dr. Donald Dean (Ohio State University, Columbus, USA) at 1:1000 dilution at 4°C for overnight following blocking of membrane with 0.5% BSA in TBS containing 0.05% Tween-20. After washing the blot for 3 x 5 minutes with TBST (TBS +0.05% Tween 20), blot was incubated with anti-rabbit IgG antibody (Sigma, Saint Louis, MO, USA) conjugated to horse-radish peroxidase at 1:10000 dilution for 2 hours at room temperature. Following three washes with TBST, detection was completed by chemiluminescence using luminol (0.4 mM) and iodophenol (8.0 mM) (Sigma, Saint Louis, MO, USA) (173).
5.2.7 Bioassay on mosquito larvae

Bioassays with transgenic Chlamydomonas carrying 3HKT inverted repeats were carried out in the laboratory of Dr. Brenda Beerntsen (University of Missouri, Columbia, MO, USA). For bioassays, freshly emerged (less than 24 hours old) larvae of Anopheles stephensi were used. Actively growing log phase culture of transgenic Chlamydomonas clones (OD\textsubscript{750} = 0.7 to 1.2) growing in TAP medium were used as inoculum for the bioassay. Bioassays were started in 12 well plates, carrying 2.0 mL of 10\% TAP medium supplemented with arginine (100 µg/mL) in each well. Each well was inoculated with 10\% inoculum of log phase cultures of transgenic Chlamydomonas clones along with 1/3\textsuperscript{rd} of the yeast-sera micron mosquito larval diet mix (1/3\textsuperscript{rd} level of the yeast-sera micron diet that is normally used for rearing mosquito larvae) and 10 freshly emerged larvae were transferred to each well. Experiments were carried out in duplicates. Observations on growth, behavior and mortality, if any of the larvae were recorded everyday. Fresh inoculum of Chlamydomonas and yeast-sera micron diet were added to wells every third day during the course of the experiment. Third instar larvae were transferred to 6 well plates, with each well containing 5.0mL of 10\% TAP.

5.3 Results

5.3.1 Cry4Ba protein is not stable in Chlamydomonas chloroplast

Cry4Ba coding sequence was cloned into the chloroplast transformation vector pBA155 creating plasmid pCry4. CC741 strain of Chlamydomonas was transformed with pCry4 using the gene gun. Transgenic colonies were selected based on expression of psbA gene that restored the photosynthetic phenotype in CC741 strain allowing the
transgenic colonies to grow on HS media. Integration of Cry4Ba coding sequence into the chloroplast genome was confirmed by PCR (Figure 5.1). The PCR positive clones were subjected to immunoblot analysis using anti Cry4Ba antibody to verify expression of Cry4Ba expression. However, instead of the expected 120kDa Cry4Ba band, a band of about 30kDa was seen in all the clones (Figure 5.2). This indicates that perhaps the Cry4Ba protein is not stable in Chlamydomonas chloroplast and the 30kDa fragment represents the degradation product of Cry4Ba.
Figure 5.1: Sketch of plasmid pCry4 and PCR analysis of CC741/pCry4 clones.

A) Plasmid map of pCry4 used for expression of Cry4Ba in Chlamydomonas chloroplast.

B) PCR analysis of CC741/pCry4 clones to confirm integration of Cry4Ba coding sequence into the chloroplast genome. Band of expected size (1.0kb) was seen in 10 clones. Wild type control (C) and plasmid control (PC), 1.0kb plus DNA ladder (M).
Figure 5.2: Immunoblot analysis of CC741/pCry4 clones.

Immunoblot analysis of PCR positive CC741/pCry4 clones. Cells equivalent to one milliliters of OD$_{750}$=1 were loaded from each culture. Blot was probed with anti Cry4Ba antibody (1:1000 dilution). Dual color precision plus protein ladder was used.
5.3.2 *Cry4Ba* expression could not be confirmed among *Chlamydomonas* nuclear transformants.

*Cry4Ba* coding sequence was cloned into *Chlamydomonas* nuclear transformation vector pSSCR7 under the control of B2-Tubulin promoter creating plasmid pCry9. Cotransformation of CC424 strain of *Chlamydomonas* was performed with the plasmids pCry9 and ARG7-encoding plasmid, p389 and transgenic colonies were selected on TAP medium. Integration of *Cry4Ba* coding sequence into *Chlamydomonas* nuclear genome was confirmed by PCR (Figure 5.3). To test expression of *Cry4Ba* protein by these PCR positive clones, immunoblot analysis was carried out using anti *Cry4Ba* antibodies. No band representing 120kDa *Cry4Ba* protein was seen among any of the PCR positive clones indicating that either these clones don’t express the *Cry4Ba* protein or the protein is not stable. Reverse-transcriptase PCR (RT-PCR) reactions were set to check presence of *Cry4Ba* transcripts among PCR positive clones. However, we failed to detect any *Cry4Ba* transcripts. The possible reason for non expression of *Cry4Ba* among *Chlamydomonas* nuclear transformants appears to be *Cry4Ba* coding sequence itself. Since *Cry4Ba* coding sequence was not codon optimized for *Chlamydomonas* nuclear expression, it can probably result in inefficient transcription and translation of *Cry4Ba*. Being a gene of prokaryotic origin, *Cry4Ba* is AT rich (64.38%), while the *Chlamydomonas* nuclear genome is GC rich (64%). This perhaps results in issues with transcription of *Cry4Ba* coding sequence. The AT rich regions of *Cry4Ba* are probably recognized as putative transcription termination signals by *Chlamydomonas* transcription
machinery leading to premature termination of transcription resulting in production of truncated transcripts.
Figure 5.3: Sketch of plasmid pCry9 and PCR analysis of CC424/pCry9 clones.

A) Map of plasmid pCry4, used for expression of Cry4Ba in Chlamydomonas nuclear genome. B) PCR analysis of CC741/pCry4 clones to confirm integration of Cry4Ba coding sequence into the nuclear genome of Chlamydomonas. Wild type control (C) and plasmid control (PC), 1kb plus DNA ladder (M).
5.3.3 Nuclear transformation of Chlamydomonas with inverted repeat constructs of Anopheles 3HKT

A 328bp region of Anopheles gambiae 3HKT gene not showing alignment of more than 11 contiguous bases to any sequence from other organisms in NCBI database was selected for creating 3HKT inverted repeat constructs. Three inverted repeat constructs were made for Chlamydomonas nuclear transformation (Figure 5.4). Construct pCVAC150 has 3HKT inverted repeat transcription under the control of Chlamydomonas Actin promoter. In construct pCVAC153, Chlamydomonas actin intron1 forms the spacer region between the HKT inverted repeat regions and the transcription of the inverted repeat is regulated by the psaD promoter. Construct pCVAC145 has the 328 bp region of 3HKT flanked by the psaD and Actin promoters from either ends, leading to bidirectional transcription of the 3HKT fragment. CC424 strain of Chlamydomonas was transformed with the above mentioned three constructs. Transgenic colonies were subjected to PCR analysis to confirm integration of the 3HKT inverted repeat in the nuclear genome of Chlamydomonas with all the three constructs (Figure 5.5, 5.6 and 5.7). These PCR positive clones were then used to carryout bioassays on mosquito larvae.
Figure 5.4: Sketch of HKT inverted repeat constructs.

Sketch of the three HKT inverted repeat constructs to produce double stranded HKT RNAs from Chlamydomonas nuclear genome. In construct pCVAC150 expression of HKT inverted repeat construct is driven by Chlamydomonas actin promoter. In construct pCVAC153 the HKT inverted repeat has Chlamydomonas actin intron1 as the spacer and the expression of the HKT inverted repeat is driven by Chlamydomonas psaD promoter. Construct pCVAC145 has expression of HKT driven by Chlamydomonas psaD and Actin promoters from either ends resulting in bidirectional transcription of HKT.
PCR analysis of CC424/pCVAC150 clones to check integration of HKT inverted repeat construct in the nuclear genome of Chlamydomonas. Binding sites of the four primers used for PCR confirmation are shown on the sketch of pCVAC150. A) Result of PCR reaction with primers 1 and 2. B) Result of PCR reaction with primers 3 and 4 on selected clones. Wild type control (C), water control (-ve) and plasmid control (+ve) 1kb DNA ladder (M).

Figure 5.5: PCR analysis of CC424/pCVAC150 clones.
Figure 5.6: PCR analysis of CC424/pCVAC153 clones.

PCR analysis of CC424/pCVAC153 clones to check integration of 3HKT inverted repeat construct in the nuclear genome of Chlamydomonas. Binding sites of the four primers used for PCR confirmation are shown on the sketch of pCVAC153. A) Result of PCR reaction with primers 1 and 2. B) Result of PCR reaction with primers 3 and 4 on selected clones. Wild type control (C), water control (-ve) and plasmid control (+ve), 1kb DNA ladder (M) N.
Figure 5.7: PCR analysis of CC424/pCVAC145 clones.

PCR analysis of CC424/pCVAC145 clones to check integration of $3HKT$ construct in the nuclear genome of Chlamydomonas. Binding sites of the two primers used for PCR confirmation are shown on the sketch of pCVAC145. Wild type control (C), water control (-ve) and plasmid control (+ve), 1kb DNA ladder (M).
5.3.4 Chlamydomonas chloroplast transformation with 3HKT inverted repeat construct.

To the best of our knowledge, the Chlamydomonas chloroplast lacks the machinery for processing of double stranded RNA (dsRNA) into siRNA. Therefore, we expect that the dsRNAs produced by Chlamydomonas chloroplast transformants would be available for processing by the RNAi machinery found in mosquito larvae. Hence we wanted to test the difference in the effect of 3HKT siRNAs produced by nuclear transformants to dsRNAs produced by chloroplast transformants on mosquito larvae. An inverted repeat construct of 3HKT was made for transcription in Chlamydomonas chloroplast (Figure 5.8). The 3HKT inverted repeat was cloned downstream of atpA promoter in pBA155 vector creating plasmid pCVAC108. Chloroplast transformation of CC4147 strain of chlamydomonas was performed with construct pCVAC108 using gene gun. Integration of the 3HKT inverted repeat into chloroplast genome was confirmed by PCR (Figure 5.8). These PCR positive clones were tested in bioassays on mosquito larvae.
Figure 5.8: Sketch of Construct pCVAC108 and PCR analysis of CC4147/pCVAC108 clones.

A) Sketch of construct pCVAC108 used to express HKT inverted repeat in Chlamydomonas chloroplast genome. Binding sites of primers used for PCR analysis are also shown. B) Results of PCR reaction to confirm integration of HKT inverted repeat expression cassette. Wild type control (C), water control (-ve) and plasmid control (+ve), 1kb plus DNA ladder (M).
5.3.5 Bioassay of HKT dsRNA producing Chlamydomonas clones on mosquito larvae

Transgenic Chlamydomonas carrying HKT inverted repeats were used to carry out bioassays on larvae of *Anopheles stephensi*. Bioassay was started on the first day of larval emergence. Transgenic Chlamydomonas clones were grown to log phase ($OD_{750}=0.7\ TO\ 1.2$) in TAP medium to use as inoculum for the bioassay. Bioassay was started in 12 well plates, carrying 2.0mL of 10% TAP medium in each well. Each well was inoculated with 10% inoculum of log phase cultures of transgenic Chlamydomonas clones along with $1/3^{rd}$ level of yeast-sera micron larval diet that is used to rear mosquito larvae and 10 freshly emerged larvae (less than 24 hrs old) were transferred to each well. Each experiment was carried out in duplicates. Growth, behavior and mortality, if any of the larvae was recorded everyday. Every third day a fresh inoculums ($OD_{750}=0.8\ to\ 1.0$) of Chlamydomonas and yeast-sera micron diet were added. Third instar larvae were transferred to 6 well plates, with each well containing 5.0mL of 10% TAP. The preliminary results from the bioassay experiments look very promising. Among Chlamydomonas chloroplast transformants (CC741/pCVAC108), more than 30% of larval mortality was observed with 5/12 clones with highest mortality of 58% observed with clone CC4147/pCVAC108-15 (Figure 5.9). Out of the three nuclear constructs, most promising results were observed with pCVAC153 construct. More than 30% of larval mortality was observed with 4/6 CC424/pCVAC153 clones (Figure 5.10). Clone CC424/pCVAC153-15 appeared to be most lethal to *Anopheles stephensi* larvae with larval mortality of 53%. CC424/pCVAC150 clones were second best among nuclear
constructs in their lethality to mosquito larvae. Five out of fourteen CC424/pCVAC150 clones exhibited toxicity to Anopheles larvae (Figure 5.11). CC424/pCVAC150-4 appeared to be most toxic (43.3% toxicity). Among CC424/pCVAC145 clones, only 1/15 clones caused more than 30% larval mortality (Figure 5.12).

The larvae feeding on transgenic Chlamydomonas expressing 3HKT dsRNA exhibited different phenotypes. Larval morality was observed in bioassay experiments starting from 2\textsuperscript{nd} instar stage. While some larvae died during 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} instar, others died during pupal stage and failed to emerge as adults (Figure 5.13). Larvae that eventually died, exhibited slow growth, were smaller in size and lagged behind other larvae growing in the same treatment or in controls. However, more comprehensive study is warranted on the adults that emerged from the surviving larvae among treatments to evaluate their ability to fly and their reproductive fitness.
Figure 5.9: *Anopheles stephensi* larval mortality observed with Chlamydomonas chloroplast transformants (CC4147/pCVAC108) expressing 3-HKT dsRNAs.

Twenty newly hatched *A. stephensi* larvae were reared on algae plus 1/3\textsuperscript{rd} yeast+ sera micron (1/3\textsuperscript{rd} amount of yeast+micron mixture fed to control larvae) in 12-well plates (transferred into 6-well plates on day 7). Mosquito death and molting were recorded daily through day 12. Number of larvae/well (N) =10, number of replications = 2.
Figure 5.10: *Anopheles stephensi* larval mortality observed with CC424/pCVAC153 clones expressing 3-\textit{HKT} dsRNAs.

Twenty newly hatched *A. stephensi* larvae were reared on algae plus 1/3\textsuperscript{rd} yeast+ sera micron (1/3\textsuperscript{rd} amount of yeast+micron mixture fed to control larvae) in 12-well plates (transferred into 6-well plates on day 7). Mosquito death and molting were recorded daily through day 12. Number of larvae/well (N) =10, number of replications = 2.
Figure 5.11: *Anopheles stephensi* larval mortality observed with CC424/pCVAC150 clones expressing 3-HKT dsRNAs.

Twenty newly hatched *A. stephensi* larvae were reared on algae plus 1/3rd yeast+ sera micron (1/3rd amount of yeast+micron mixture fed to control larvae) in 12-well plates (transferred into 6-well plates on day 7). Mosquito death and molting were recorded daily through day 12. Number of larvae/well (N) =10, number of replications = 2.
Figure 5.12: *Anopheles stephensi* larval mortality observed with CC424/pCVAC145 clones expressing 3-**HKT** dsRNAs.

Twenty newly hatched *A. stephensi* larvae were reared on algae plus 1/3rd yeast+ sera micron (1/3rd amount of yeast+micron mixture fed to control larvae) in 12-well plates (transferred into 6-well plates on day 7). Mosquito death and molting were recorded daily through day 12. Number of larvae/well (N) =10, number of replications = 2.
Figure 5.13: Different phenotypes exhibited by *Anopheles stephensi* larvae following feeding on transgenic algae producing 3HKT dsRNA.

This is a representative data of various kinds of phenotypic effects observed on mosquito larvae following feeding on different transgenic Chlamydomonas clones expressing HKT dsRNA. A) Dead larvae of *Anopheles stephensi* reared on transgenic algae. B) Comparison of a larva exhibiting growth inhibition (red arrow) upon feeding on transgenic algae to a normally developing larva (green arrow) on day 12. C) Image of a dead (red arrow) and a live pupae (green arrow) in an experimental well with transgenic algae. Newly hatched *A. stephensi* larvae were reared on algae plus 1/3rd yeast+ sera micron (1/3rd amount of yeast+micron mixture fed to control larvae) in 12-well plates (transferred into 6-well plates on day 7). Mosquito death and molting were recorded daily through day 12.
5.4 Discussion

5.4.1 Several issues with Cry4Ba expression in Chlamydomonas

We attempted to develop the micro-alga *C. reinhardtii* as a mosquito larvicide. Two strategies were tried. While the first strategy involved expression of Cry4Ba endotoxin from Bti in Chlamydomonas nuclear/chloroplast, the second strategy targeted silencing genes essential for mosquito survival by feeding transgenic Chlamydomonas expressing dsRNA targeting these genes to mosquito larvae. While Cry4Ba protein appeared to be unstable in Chlamydomonas chloroplast, no expression of Cry4Ba was observed from the nuclear genome. The Cry4Ba is expressed along with Cry4Aa, Cry11Aa and CytAa in Bti and all the four proteins together form the parasporal crystal in Bti (1). Therefore, it is possible that expression of Cry4Ba alone makes the protein unstable resulting in degradation of the protein in Chlamydomonas chloroplast. To stabilize Cry2Aa2 crystals and enhance its expression in tobacco chloroplasts, Cosa et al. (34) employed Cry2Aa2 operon coding a chapronin that facilitates folding of Cry2Aa2 to form stable crystals. Hence, it may be possible to stabilize expression of Cry4Ba in Chlamydomonas chloroplast by expressing it along with other toxin proteins that form parasporal crystals in Bti or chapronin proteins that could stabilize the protein.

Since we did not use codon optimized Cry4Ba coding sequence for nuclear expression, the non expression of Cry4Ba from nuclear genome perhaps stems from the composition of the coding sequence of Cry4Ba. Being a gene of prokaryotic origin, Cry4Ba is AT rich (64.38%), while the Chlamydomonas genome is GC rich (64%). The Chlamydomonas transcription machinery probably recognizes the AT rich regions of
Cry4Ba as putative transcription termination signals resulting in production of unstable truncated transcripts. Similar problems were encountered during initial attempts to express Cry proteins in plants to develop insect resistance (11, 40). Truncated and unstable transcripts of Cry1Ac were seen when the AT rich native coding sequence was used for expression in tobacco, while use of a synthetic GC rich Cry1Ac in tobacco plants led to production of full length stable transcripts resulting in good expression of protein (35). Thus, the problem of non expression of Cry4Ba from nuclear genome of Chlamydomonas can perhaps be overcome by codon optimization of Cry4Ba for nuclear expression.

5.4.2 Inhibition of 3HKT expression is an effective strategy for mosquito control

3HKT is a unique protein found only in mosquitoes. This protein has evolved in mosquitoes to compensate for the absence of KAT found in humans and mammals. 3HKT catalyzes transamination of the reactive 3HK to more stable XA in tryptophan metabolic pathway (67). Hence knocking down expression of 3HKT could lead to accumulation of 3HK, which can be lethal to mosquitoes. Moreover since 3HKT is expressed during the active feeding stages of mosquitoes (136), we explored the possibility of knocking down 3HKT by feeding transgenic Chlamydomonas expressing 3HKT dsRNA. Feeding of transgenic Chlamydomonas expressing 3HKT inverted repeats either from chloroplast or nuclear genome was found to be effective in inhibiting growth of mosquito larvae and ultimately causing their death. CC4147/pCVAC108 (chloroplast transformants) and CC424/pCVAC153 clones (nuclear transformants) appeared to most toxic to mosquito larvae. More than 50% larval mortality was observed with two clones of
CC4147/pCVAC108 and one clone of CC424/pCVAC153. Larval mortality in the range of 30-40% was also observed with CC424/pCVAC150 and CC424/pCVAC145 clones.

The chloroplast transformants producing 3HKT dsRNAs appear to be more effective in inhibiting mosquito larvae when compared to nuclear transformants. Perhaps the packaging of the 3HKT dsRNA in the plastid provides extra protection and prevents it from degradation during ingestion. The CC424/pCVAC153 nuclear transformants were expected to perform better than other nuclear constructs because expression of 3HKT inverted repeat in pCVAC153 is driven by the psaD promoter, the best promoter we have found for transgene expression in Chlamydomonas. Inclusion of functional introns as the spacer element in the IR constructs are known to enhance efficiency of gene silencing (93, 149). Since the pCVAC153 construct has Chlamydomonas actin intron1 as the spacer element between the 3HKT IR, it perhaps enhances the expression and stability of 3HKT dsRNA contributing to efficient 3HKT silencing.

In brief, the preliminary results from the bioassay experiments show that dsRNAs targeting essential mosquito genes produced in Chlamydomonas can be used as an effective strategy to control mosquito populations. The results look very promising and the efficiency of this system can perhaps be further improved by targeting two or more genes. However, experiments need to be done to prove that the larval mortality observed upon feeding transgenic Chlamydomonas producing 3HKT dsRNAs is due to knocking down of 3HKT expression in mosquito larvae.
CHAPTER 6: SUMMARY

The objective of this research work was to demonstrate the potential biotechnological applications of the microalga *Chlamydomonas reinhardtii*, which range from production of biofuels, hydrogen, and recombinant proteins, such as biopharmaceuticals. *C. reinhardtii* was selected because 1) it grows fast 2) has low production costs 3) is a model genetic organism which can be easily transformed 4) being a eukaryote, it has the machinery for post translational modification of proteins 5) availability of mutant strains that allow secretion of the protein into the medium facilitating protein purification 6) it is considered as a safe food-grade material 7) it is non-toxic and non-pathogenic, and 8) stable transgenic lines can be generated in less than 3 weeks and production can be scaled up in a few months. Even though transgenes can be expressed from any of the three genomes of Chlamydomonas, only protein production from the single large chloroplast of Chlamydomonas has been shown to be of economic significance. Much needs to be done to make protein production from Chlamydomonas nuclear genome to be cost effective. Hence, we attempted to optimize transgene expression from Chlamydomonas nuclear genome.
Chapter 2 provides an in depth analysis of factors that affect transgene expression in Chlamydomonas. Five Chlamydomonas promoters (B2-Tubulin, Fea1, psaD, Actin and Hsp70A/Rbcs2) and two heterologous viral promoters (Chlorella virus DNA polymerase promoter (CVDP) and Cauliflower mosaic virus 35S (CaMV35S)) were evaluated for transgene expression in Chlamydomonas. Effect of three different terminator regions from Chlamydomonas (CCP1, B2-Tubulin and psaD) on transgene expression and factors that affect Chlamydomonas transformation efficiency and stability of transgene expression were also evaluated.

Luciferase from Gaussia princeps was employed as reporter to compare the strength of selected promoters and effects of other elements that were tested in this study. The Chlamydomonas psaD promoter was found to be superior in terms of transgene expression in Chlamydomonas compared to the other 6 promoters used in this study. The Chlamydomonas Actin and the widely used Hsp70A/Rbcs2 fusion promoters came second and third respectively. The fact that the psaD gene codes for a gene that lacks introns, possibly contributes towards its ability to drive excellent expression from transgenes lacking introns (54). The heterologous viral promoters performed poorly, perhaps due to significant differences in the GC content of these promoters and the Chlamydomonas genome which probably leads to poor initiation of transcription from these viral promoters by the Chlamydomonas transcriptional machinery. However, the unexpected finding of this study was the effect of terminator on transgene expression, which was much stronger than the effect of promoters.
The transcription terminator plays a very important role in gene expression as it determines the stability of transcript, nuclear to cytoplasmic transport and translation competence of the transcript (178). However not much work has been done to compare and identify an optimal terminator for transgene expression in either plants or Chlamydomonas. The effect of terminator regions of Chlamydomonas CCP1, B2-Tubulin and psaD on luciferase expression was studied. These three terminators were used in combination with B2-Tubulin and psaD promoters. Highest luciferase expression was observed among clones carrying psaD terminator. Clones carrying psaD terminator had 6 to 10 fold higher luciferase expression than clones carrying the luciferase gene driven by the same promoter but flanked by a different terminator. The implication being that the psaD terminator is perhaps more efficient at controlling transcription termination and 3’ end processing of mRNA when compared to B2-Tubulin or CCP1 terminators, resulting in higher luciferase expression. Studies with different terminators in plants have also reported similar observations (76 111). Notably, the psaD terminator was not only effective in improving transgene expression in combination with its endogenous psaD promoter but also with the heterologous B2-Tubulin promoter. When used in combination with B2-TubulinP, the psaD terminator increased luciferase expression by 10-fold when compared to the B2-TubulinP in combination with B2-Tubulin terminator construct. This indicates that the psaD terminator can be used in combination with other promoters to improve transgene expression.

An efficient terminator enhances gene expression by improving the stability of transcripts. Evaluation of luciferase transcript levels among single copy transformants of
pCVAC117 (psaDP-LUC-CCP1 ter) and pCVAC157 (psaDP-LUC-psaD ter) by real time PCR showed that the pCVAC157 clones had 2 to 10-fold higher luciferase transcripts when compared to pCVAC117 transformants. This demonstrates that the psaD ter enhances luciferase expression by increasing the steady state levels of luciferase transcripts. Another important finding of this study was that the state of the plasmid used for Chlamydomonas transformation significantly affects transformation efficiency and the percentage of clones showing transgene expression. Chlamydomonas transformation was performed with either supercoiled plasmid/linear plasmid or expression cassettes (selection marker and gene of interest together). The transformation efficiency with expression cassette was 2.35 fold higher than that observed with supercoiled plasmid. The percentage of clones showing luciferase expression also increased from 32% with supercoiled plasmid to 77% with expression cassettes. Similar observations were also made by Kindle (85), however they used only linearized plasmid for their studies. The linearized plasmid/expression cassette perhaps increases transformation efficiency by facilitating integration of transgene into the genomic DNA of Chlamydomonas over supercoiled plasmid resulting in better transformation efficiency.

The stability of luciferase expression in Chlamydomonas was also evaluated as there have been conflicting reports regarding stability of transgene expression in Chlamydomonas. The results indicate that transgene expression is relatively stable in Chlamydomonas provided that the gene of interest and the selection marker are driven by different promoters. The results also demonstrate that transgene silencing in Chlamydomonas appears to be mostly established during initial stages/ generations of a
transgenic colony and the clones that are not affected by transgene silencing during initial stages remain relatively stable and continue to maintain good expression levels of the transgene.

Overall the above mentioned results indicate that several factors affect transgene expression in Chlamydomonas and the findings of the above mentioned work can be employed to significantly enhance transgene expression from nuclear genome of Chlamydomonas.

Chapter 3 demonstrates the potential of *C. reinhardtii* as a biofactory for production of therapeutic proteins. Two proteins of therapeutic value, human paraoxonase1 (*PON1*) and AHL lactonase from bacteria were expressed in Chlamydomonas and were found to be functional. *PON1* is anti-atherosclerotic (150) and can also hydrolyze organophosphate (OP) (nerve gases) substrates into biologically inert products (90, 94). The AHL lactonase (*AiiA*) enzyme produced by various *Bacillus* *sps*, disrupts the quorum sensing (QS) among gram negative bacteria by destroying the QS signal molecules N-acyl homoserine lactones (AHL) (41, 43).

*PON1* remains associated with HDL through ApolipoproteinA1 (*ApoA1*) in blood serum (160). In the absence of *ApoA1*, *PON1* becomes highly hydrophobic and toxic to host cells. For the same reason, expression of *PON1* in bacterial and other expression systems is problematic. To date the only way to produce paraoxonase1 for therapeutical applications is through purification from mammals, which makes its production very expensive. Therefore, we explored the possibility of expressing *PON1* in Chlamydomonas. Expression of *PON1* and its variants was observed in Chlamydomonas...
nuclear as well as chloroplast transformants. PON1 expressed in Chlamydomonas appeared to be insoluble since the paraoxonase activity was observed in the insoluble fraction of the cell lysate. Interestingly however, expression of PON1 and its variants was not toxic to Chlamydomonas cells as transgenics have survived for more than 2 years now. This is a very promising result because till date no transgenic plant expressing PON1 has been recovered due to toxicity associated with expression of PON1. Furthermore, fusion of luciferase to the C-terminus of PON1 (PON1-luciferase) appeared to improve the solubility of the protein as some amount of the fusion protein was visible in the soluble fraction as indicated in immunoblot experiments. It would be of interest to express PON1 along with ApoA1 in Chlamydomonas to see whether this improves the solubility and activity of PON1.

With the ever increasing problem of development of antibiotic resistance among bacterial species, new strategies to control bacterial infections are being explored. One of the most sought after strategies involves controlling bacterial infections by interfering with the quorum sensing (QS) mechanism found in bacteria (127). AHL lactonase found in various Bacillus sps inactivates AHLs, the quorum sensing signal molecules and interfere with QS. In fact AHL lactonase expressing plants were found to be resistant to bacterial infection (41). Furthermore, compounds that mimic the bacterial QS signals also interfere with QS. The marine alga Delisea pulchra produces halogenated furanones that act as an AHL mimic and bind to AHL receptor polypeptides. This causes receptor misfolding and proteolytic degradation, resulting in inhibition of the QS signaling pathway in bacteria (63, 100). Since Chlamydomonas is known to secrete CepR mimics
(161), an attempt was made to find out whether these mimics have any structural similarity to AHLs by subjecting the mimics to AHL lactonase treatment. We observed a significant reduction in the activity of Chlamydomonas AHL mimics upon treatment with AHL lactonase, indicating that these mimics may have a lactone ring in common with AHLs. This also suggests that the mimics produced by Chlamydomonas could have potential pharmaceutical value similar to the halogenated furanones produced by Delisea pulchra. Transgenic Chlamydomonas expressing AHL lactonase-CFP fusion showed a significantly lower mimic activity when compared to controls, indicating that the AHL lactonase produced by these transgenics was functional and was perhaps responsible for the reduced levels of mimics in these transgenics. More experiments are warranted to study the effect of reduction in production of AHL mimics by transgenic Chlamydomonas expressing AHL lactonase-CFP on algal growth, their sensitivity to bacterial contamination or interactions with bacteria in their natural habitat.

Overall we have shown that Chlamydomonas can be used to produce difficult eukaryotic proteins such as human PON1. Human PON1 and AHL lactonase produced in Chlamydomonas were functional and the QS mimic compounds produced by Chlamydomonas perhaps share lactone ring with AHLs. More experiments need to be done to optimize expression of human PON1 in Chlamydomonas and to study the role of QS mimics produced by Chlamydomonas.

Chapter 4 demonstrates the potential of C. reinhardtii as a system for oral delivery of vaccines to fish, animals and possibly humans. Here an attempt was made to develop the unicellular alga, C. reinhardtii as a system to deliver vaccines to fish against
infectious hematopoietic necrosis virus (IHNV). Glycoprotein (G), found on the surface of IHNV particles is known to induce production of neutralizing antibodies in rabbits and fish (50). Based on this, two strategies to develop algal based vaccines against IHNV were evaluated. The first strategy was an epitope based strategy that involved identification of putative G protein epitopes and expression of selected epitopes as C-terminus fusions with the p57 protein of *Renibacterium salmoninarum* in Chlamydomonas chloroplast. Attempts have been made in past to map the neutralizing epitopes of G protein using monoclonal antibodies (73, 171) and it has been shown that fish vaccinated with lysate of bacterial cells expressing fragments of G protein provide effective protection to fish (171). Therefore, a dodecapeptide phage display library was screened against trout anti-IHNV immune serum to identify phage sequences that mimic IHNV-G epitopes. Importantly, the screen was able to pick the known linear epitope of G protein as 3 phage sequences showed alignment with it. As expected, the G protein conformational epitope was not picked. All the remaining phages aligned to the predicted surface residues of the G protein. Transgenic Chlamydomonas expressing putative G protein epitopes fused to C-terminus of p57 were used to vaccinate rabbits orally and the immune serum from rabbits was analyzed for presence of anti-G protein antibodies by ELISA and immunoblot. However, we failed to detect synthetic peptides representing the putative epitopes in ELISA or the G protein in immunoblots with the immune serum from rabbits. This indicates that either the selected epitopes did not induce any immune response in rabbits or the antibodies in the immune serum could not recognize the synthetic peptides or G protein. The result was not surprising as there are no success
stories as far as epitope based vaccines are concerned. Although several epitope based vaccines against HIV, Hepatitis C and Influenza virus are in advanced stages of trials (2, 78, 132, 176). Previous attempts of intraperitoneal injection of trout with synthetic peptides representing putative G protein epitopes conjugated with bovine serum albumin and mixed with adjuvant had also failed to provide any protective immunity (49). Incorrect epitope prediction, use of inappropriate carrier molecule and lack of T-cell stimulation by the selected peptides are probably the possible reasons for failure of putative G protein epitopes to induce immune response in the present and earlier studies.

The second strategy involved expression of G protein in Chlamydomonas. This strategy was driven by the earlier reports of successful vaccination of fish with cell lysate from bacterial cells expressing fragments of G protein (171). Immune serum from rabbits fed with transgenic Chlamydomonas expressing luciferase-G protein fusions was able to detect G-Tobacco mosaic virus coat protein fusion (TMVCP) protein in the immunoblots indicating that the serum contained anti-G protein antibodies and that this is a viable strategy. The next step to validate this strategy is to use the transgenic Chlamydomonas expressing luciferase-G protein fusions to conduct a vaccination trial on fish. Again the prospects of success with this approach are high because similar studies involving oral vaccination of salmonid fish with yeast cells expressing viral capsid protein of infectious pancreatic necrosis (IPN) virus were able to induce specific immune response in fish (4).

In brief, in this work we demonstrated that feeding of transgenic Chlamydomonas expressing IHNV G protein to rabbits induces specific immune response in rabbits against G protein. These transgenic algae will now be used to carry out a vaccination trial
on fish. This same strategy can be potentially used to orally vaccinate animals and possibly humans against important viral diseases.

Chapter 5 demonstrates yet another biotechnological application of *C. reinhardtii*. Here we attempted to develop the micro-alga *C. reinhardtii* as a mosquito larvicide. Two strategies were tried. While the first strategy involved expression of *Cry4Ba* endotoxin from *Bacillus thuringiensis* (Bti) in Chlamydomonas nuclear/chloroplast, the second strategy involved silencing of genes essential for mosquito survival by feeding transgenic Chlamydomonas expressing dsRNA targeting these genes to mosquito larvae.

While *Cry4Ba* protein appeared to be unstable in Chlamydomonas chloroplast, no expression of *Cry4Ba* was observed from the nuclear genome. The *Cry4Ba* is expressed along with *Cry4Aa, Cry11Aa* and *CytAa* in Bti and all the four proteins together form the parasporal crystal in Bti (1). Therefore, it is possible that expression of *Cry4Ba* alone makes the protein unstable resulting in degradation of the protein in Chlamydomonas chloroplast. The non expression of *Cry4Ba* from nuclear genome is perhaps due to the composition of the coding sequence of *Cry4Ba*. Being a gene of prokaryotic origin, *Cry4Ba* is AT rich (64.38%), while the Chlamydomonas genome is GC rich (64%). The Chlamydomonas transcription machinery probably recognizes the AT rich regions of *Cry4Ba* as putative transcription termination signals resulting in production of unstable truncated transcripts. Similar problems were encountered during initial attempts to express Cry proteins in plants to develop insect resistance (40). Truncated and unstable transcripts of *Cry1Ac* were seen when the AT rich native coding sequence was used for expression in tobacco, while use of a synthetic GC rich *Cry1Ac* in tobacco plants led to
production of full length stable transcripts resulting in good expression of protein (35). Thus, the problem of non expression of Cry4Ba from nuclear genome of Chlamydomonas can perhaps be overcome by codon optimization of Cry4Ba for nuclear expression.

3-Hydroxykynurenine transaminase (3HKT) is a unique protein found only in mosquitoes. This protein has evolved in mosquitoes to compensate for the absence of Kynurenine aminotransferases (KAT) found in humans and mammals. 3HKT catalyzes transamination of the reactive 3HK to more stable XA in tryptophan metabolic pathway (67). Hence knocking down expression of 3HKT could lead to accumulation of 3HK, which can be lethal to mosquitoes. Since 3HKT is expressed during the active feeding stages of mosquitoes (136), we explored the possibility of knocking down 3HKT by feeding transgenic Chlamydomonas expressing 3HKT dsRNA. Preliminary results show that feeding of transgenic Chlamydomonas expressing 3HKT inverted repeats either from chloroplast or nuclear genome was toxic to mosquito larvae. CC4147/pCVAC108 (chloroplast transformants) and CC424/pCVAC153 clones (nuclear transformants) appeared to most lethal to mosquito larvae. Anopheles larvae fed on transgenic Chlamydomonas exhibited a range of phenotypes such slow growth, smaller size and death either during different larval or pupal stage.

In brief, the preliminary results strongly support the idea that dsRNAs targeting essential mosquito genes produced in Chlamydomonas can be used as an effective strategy to control mosquito populations. However, experiments need to be done to
demonstrate that feeding of transgenic algae expressing ds3HKT RNA to mosquito larvae results in knocking down expression of $3HKT$. 
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